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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	In progress
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	28-48	In progress
SA3b. Integrated transcriptomics	28-48	In progress
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
Previously, we reported on the development of isogenic MPNST cell lines that we have used to interrogate drug sensitivity and signaling adaptations. The results identified several notable pathways of drug resistance that we have continued investigating. In this grant period, we continued 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.

<u>p53 Transcriptionally Regulates MET Stability and Localization</u>

Receptor tyrosine kinase (RTK) expression, activation, and recycling are tightly regulated processes that ensure RTK regulation in normal physiological conditions. Deregulation of this cycle of receptor activation and recycling is often observed in cancers, such as the MET-exon 14 deletions observed in lung cancers. Thus, we sought to determine if loss of p53 altered the kinetics of MET activation and turnover to increase effector activation. In

NF1-MET cells we observed, as expected, an immediate increase in MET activation within 5 minutes of HGF

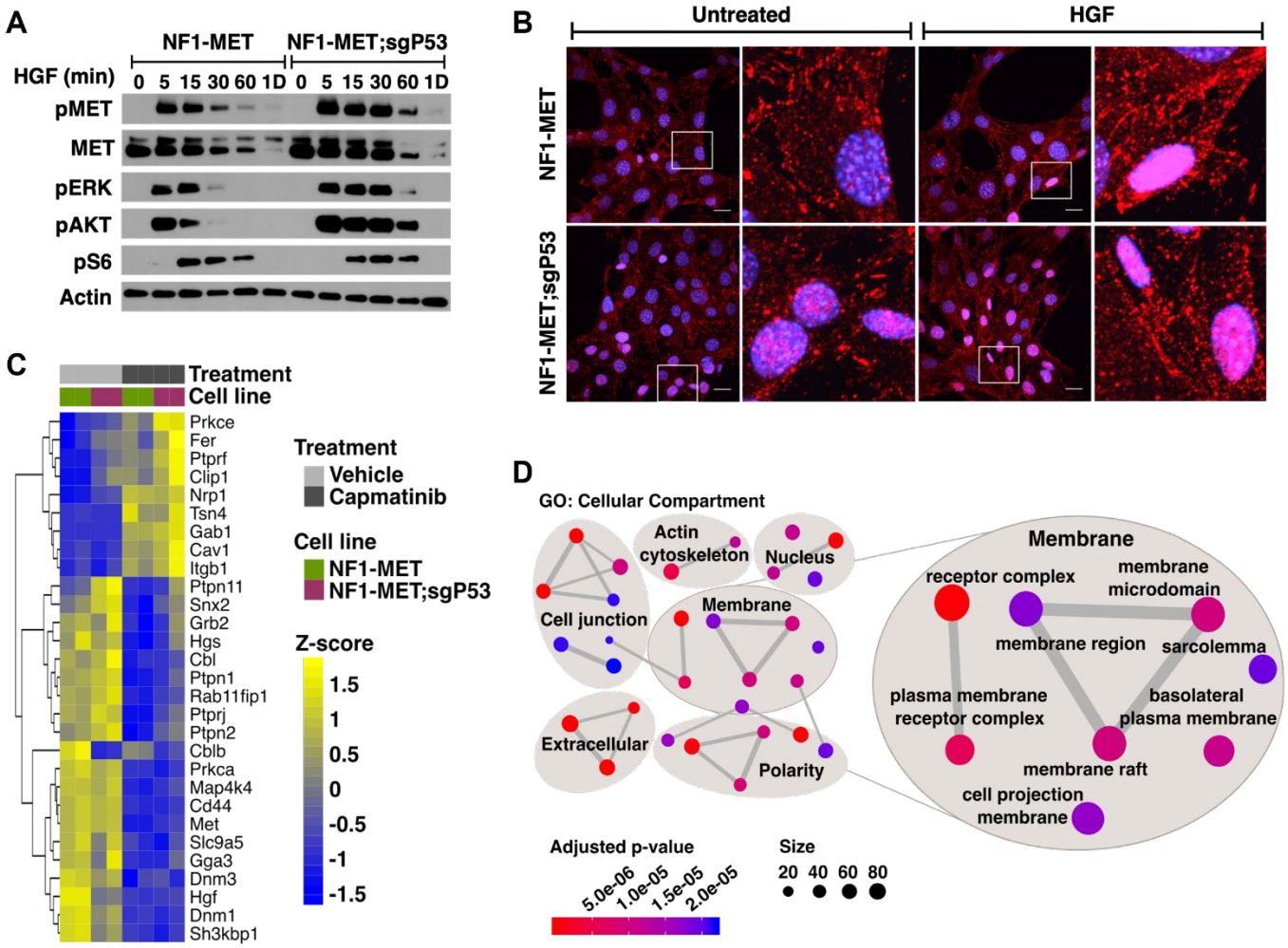

Figure 1. **p53 regulates MET stability, localization, and effector signaling.** (A) Time course western blot of NF1-MET and NF1-MET;sgP53 cells stimulated with HGF for 5 minutes to 1 day. (B) Representative images of phospho-MET localization (red) after 5 minutes of HGF stimulation. (C) Expression of genes associated with MET stability and localization after 24 hours of vehicle (DMSO) or capmatinib (100 nM) treatment. (D) GO analysis of Cellular Compartment terms downregulated in the NF1-MET;sgP53 cell line compared to parental NF1-MET line.

treatment that quickly diminished along with total MET levels over time (Figure 1A). In contrast, in the NF1-MET;sgP53 cells HGF treatment induced a drastic increase in pMET (phosphorylated MET) at 5-60 minutes compared to NF1-MET cells. Both phosphorylated and total MET were persistently elevated in the NF1-MET;sgP53 cells, which corresponded to increased and prolonged activation of both ERK and AKT (Figure 1A). Activation of the mTOR effector pS6 was similar between the two cell lines. To examine whether p53 loss also alters MET localization, we performed immunostaining of MET after HGF-treatment. Typically, HGF induces pMET at the cell membrane, however MET can also be internalized to the nucleus, where it's function is incompletely understood. At baseline, pMET was localized to the cytoplasm in the NF1-MET cells, compared to the nuclear and cytoplasmic staining observed in the untreated NF1 MET;sgP53 cells. Interestingly, HGF dramatically increased nuclear MET localization in the p53 deficient NF1 MET;sgP53 cells, while treatment induced nuclear MET localization only in a small percentage of NF1-MET cells (Figure 1B). In several cancers, nuclear MET is associated with drug resistance and poor prognosis.[1-3] These results indicate that p53 loss in MPNST cells may induce nuclear MET localization, promoting tumor aggressive phenotypes.

To understand how loss of p53 promotes increased stability and nuclear localization of MET, we used RNA-seq to examine the expression of genes involved in MET activation and turnover. As expected, MET inhibition by the MET-inhibitor capmatinib induced sweeping compensatory expression changes in both the NF1-MET and NF1-MET;sgP53 cell lines (Figure 1C). Somewhat unexpectedly, baseline Hgf expression was actually decreased in the NF1-MET;sgP53 cells, suggesting a possible explanation for their nonautonomous growth advantage.

Interestingly, expression of Sh3kpb1 and Cblb, which are critical for degradation of activated MET.[4-6] were significantly downregulated in the p53 knockout cells at baseline. Conversely, Prkce expression, which is required for nuclear MET translocation[7] was significantly upregulated in the NF1-MET;sgP53 cells compared to the parental NF1-MET cell line upon MET inhibition (Figure 1C). Further GO Cellular Compartment enrichment network analysis specifically of genes that were downregulated by p53 loss identified significant changes in plasma membrane and receptor organization pathways (Figure 1D). 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.

p53 loss drives mTOR Dependency in MPNSTs

AKT activation was consistently elevated and sustained in p53-deficient MPNST cell lines and is targetable therapeutically, either directly or via its downstream effectors. To determine the scope of AKT/mTOR pathway activation in vivo, we assessed the phosphorylation status of mTORC1 and mTORC2 pathway effectors in MPNST tumorgrafts by RPPA. Globally, phosphorylation was significantly increased in NF1-P53 tumors compared to NF1-MET tumors (p-value = 0.0034) (Figure 2A), with increased activation of 7 of 12 phosphosites in NF1-P53 tumors (Figure 2B). AKT and S6RP phosphorylation were significantly increased in the NF1-P53 tumors (Figure 2B), suggesting increased dependency on the AKT/mTOR pathway. Regardless of p53 status, treatment with the AKT inhibitor, afuresertib, had no effect on the MPNST cell lines, either as a single agent or in combination with trametinib (data not shown). However, treatment with the mTOR inhibitor, everolimus, significantly decreased viability in the p53 deficient cell lines compared to the p53 intact cells (Figure 2C). This enhanced inhibition of the p53 deficient cells was observed even though everolimus strongly inhibited downstream pS6RP expression regardless of p53 status (Figure 2D). Further, combination therapy of mTOR (everolimus) and MEK (trametinib) inhibition reversed clonal selection for p53 deficiency (Figure 2E-F).

As combination mTOR and MEK inhibition was so effective in inhibiting MPNST cell growth, we next asked whether treatment reversed the nuclear localization of MET leading to global downregulation of MAPK

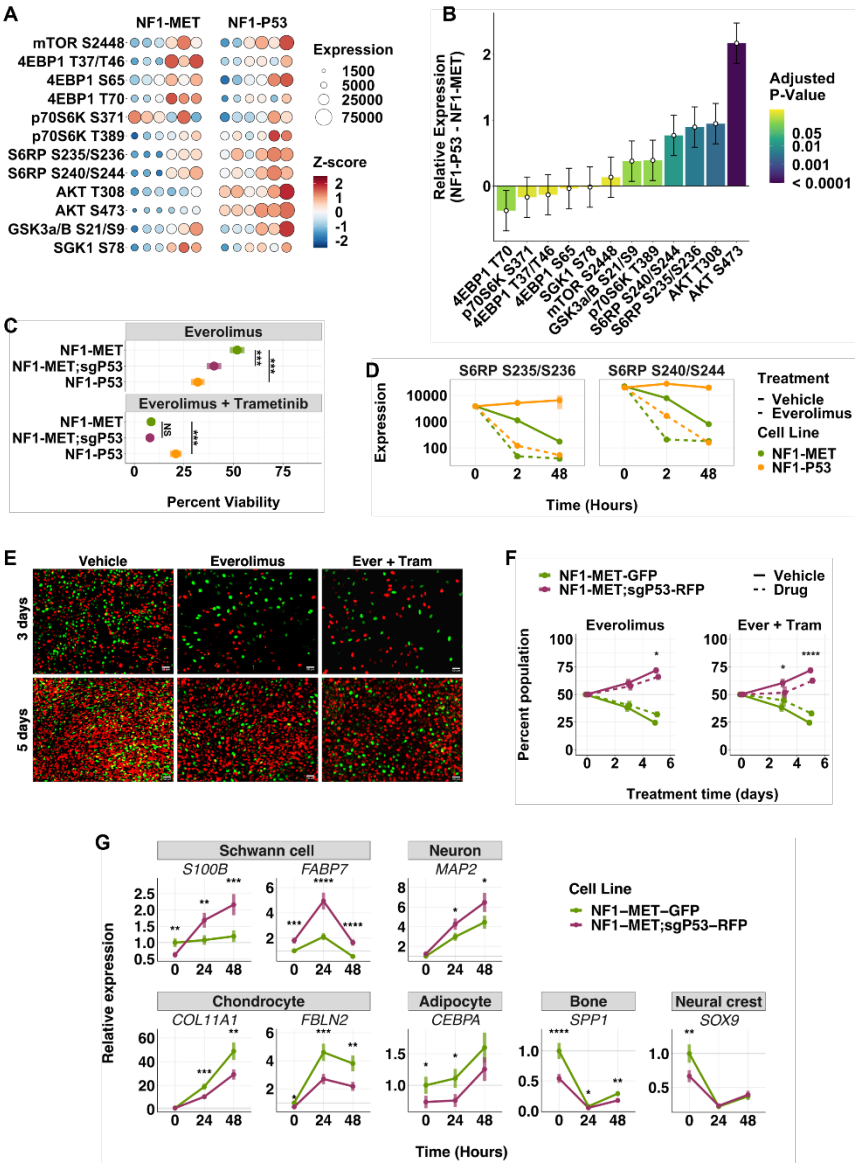

Figure 2. p53 deficiency induces mTOR dependency. (A) mTORC1/mTORC2 phospho-protein expression (size) and z - score (color) of NF1-MET and NF1-P53 tumors. (B) Contrast estimates +/- SE of mTORC1/mTORC2 phospho-protein expression in NF1-P53 tumors compared to NF1-MET tumors. Color indicates P-value. (C) Percent viability of NF1-MET, NF1-MET;sgP53, and NF1-P53 cells after 72 hours of everolimus (20 nM) or combination (everolimus 20 nM, trametinib 40 nM) treatment. (D) Phospho-S6RP expression measured over time by RPPA after vehicle (DMSO) or everolimus (100 nM) treatment. Images (E) and flow cytometry analysis (F) of GFP labeled NF1-MET and RFP labeled NF1-MET;sgP53 cells after 3 and 5 days of treatment with vehicle (DMSO), everolimus (20 nM), or combination everolimus (20 nM) and trametinib (40 nM). (G) Expression (relative to the housekeeping gene PPIA) of cell fate markers by qRT-PCR upon treatment with combination everolimus (20 nM) and trametinib (40 nM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and AKT/mTOR signaling. Unexpectedly, combination everolimus and trametinib actually induced ligand independent MET localization specifically in the NF1-MET;sgP53 cells compared to the parental line. Moreover, treatment with everolimus or the dual PI3K/mTOR inhibitor bez235 induced stronger compensatory ERK and AKT activation in NF1-P53 cells, consistent with increased MET activation (data not shown). These data reinforce the role of p53 in regulating MET localization and effector signaling in response to diverse stimuli, but also suggest that nuclear MET and increased signaling plasticity do not necessarily promote a tumor aggressive phenotype as both of the p53 deficient cell lines were more sensitive to everolimus treatment.

As differences in cell signaling, apoptosis, and senescence did not explain the increased dependency on mTOR in the p53 deficient cell lines, we next wondered if drug treatment altered cell differentiation pathways. Indeed, expression of the Schwann cell markers S100B and FABP7 were significantly increased in the NF1-MET;sgP53 cells compared to the parental cell line with combination everolimus and trametinib treatment (Figure 2G). Because we had observed evidence of transdifferentiation in capmatinib treated cells, we also surveyed markers for neuron, chondrocyte, adipocyte, bone, kidney, endothelial, and muscle cell differentiation, as well as the multipotency marker SOX9, which is expressed in neural crest cells. Interestingly, the neuronal marker MAP2 was also increased with drug treatment in the NF1-MET;sgP53 cells, while chondrocyte differentiation was strongly induced in the parental line compared the p53 null cells (Figure 2G). Adipocyte and bone differentiation markers were also differentially expressed, although to a lesser extent (Figure 2G). Consistent with the induction of multiple differentiation pathways upon mTOR and MEK treatment, the multipotency marker SOX9 was decreased in both cell lines (Figure 2G). **Collectively, these data suggest that p53 regulates cell fate determination in MPNST cells, which may partially explain differences in response to drug treatments targeting mTOR, MEK, and MET.**

Current/Future Studies: The studies in this Aim are completed and the manuscript is in preparation for submission this fall.

Specific Aim 2: Determine the efficacy of targeting MEK and AKT in p53-deficient MPNSTs.
Previously we demonstrated increased AKT activity was observed in trametinib-resistant tumors in mouse models of NF1-related MPNSTs. To determine if this adaptive kinome response was present in human MPNST cell lines, we treated MPNST cell lines NF90 and S462 with a dose response of the MEK inhibitor trametinib and performed a Western blot to evaluate the effects on AKT signaling. Western blot analysis of AKT expression and activity confirmed that this response to MEK inhibition also occurs in the human MPSNT cell lines (Figure 3A). To determine if combined AKT and MEK inhibitors would have additive or synergistic inhibition on these lines, we treated MPNST cells with trametinib and the AKT inhibitor afuresertib. In a matrix dose response assay, combining trametinib with the AKT inhibitor afuresertib, no additive effect on cell viability was seen at any combination (Figure 3). There was also no additive effect seen as measured by the Bliss synergy model (data not shown). This assay did confirm the effectiveness of MEK inhibition on these cell lines. 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 (data not shown). 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.

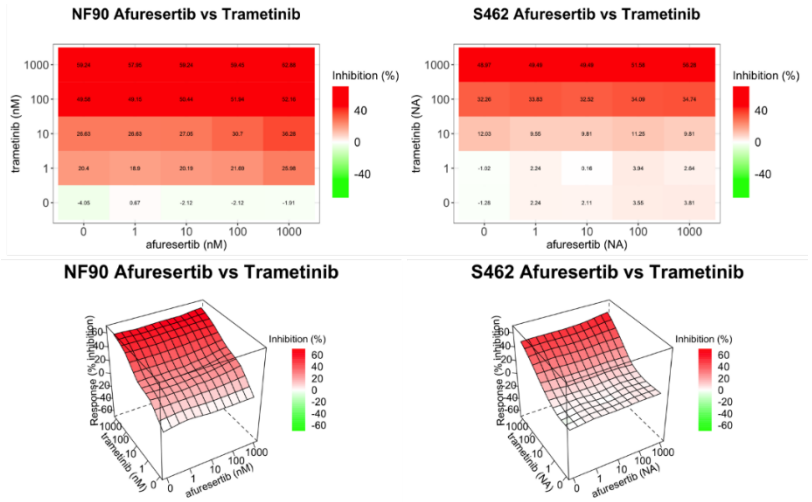

Figure 3. Combined MEK and AKT inhibition does not abrogate MPNST cell growth. Matrices of MPNST cell viability in response to combined treatment with trametinib and afuresertib. Top panel represents a heatmap of percent viability for each drug combination. The bottom panel represents a 3D visualization of the interaction between each drug combination.

Modeling MPNST treatment resistance with PDX models

To assess drivers of MPNST resistance, we have 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 are able to evaluate patterns of response and resistance to resumed treatment (Figure 4A). As shown in Figure 4A, PDX tumors from MSTA-440-2 (developed in the Steensma laboratory) were treated for 15 days with everolimus (mTOR inhibitor), trametinib (MEK inhibitor), or combined everolimus + trametinib (mTOR + MEK). After 15 days of treatment, mice were then put on a drug holiday to mimic the drug holidays that patient's experience in the clinic. Treatment was interrupted until the tumor size increased >1000 mm^3. At this point, mice resumed therapy with either everolimus and/or trametinib in a cross-over study design. 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. Across treatments, there is variable reduction in tumor growth with a few tumors that were slower growing than others. Overall, we see even in tumors that initially responded to treatment, they quickly rebound when placed onto the drug holiday. The rapid rebound of these tumors mirrors the aggressive nature of MPNST growth in patients. Variable responses are seen between several tumors on the same treatment, revealing the inherent tumor heterogeneity present in MPNSTs (Figure 4B). Continued, aggressive growth rates are seen in the vehicle control tumors, with varied responses in the initial treatment groups. Initial treatment with the combination of trametinib and everolimus suppressed initial tumor growth; however, tumors quickly rebounded after treatment cessation and only one tumor, which was placed back onto the combination, responded to secondary treatment. Mice originally placed on everolimus did not see a large reduction of tumor volume and

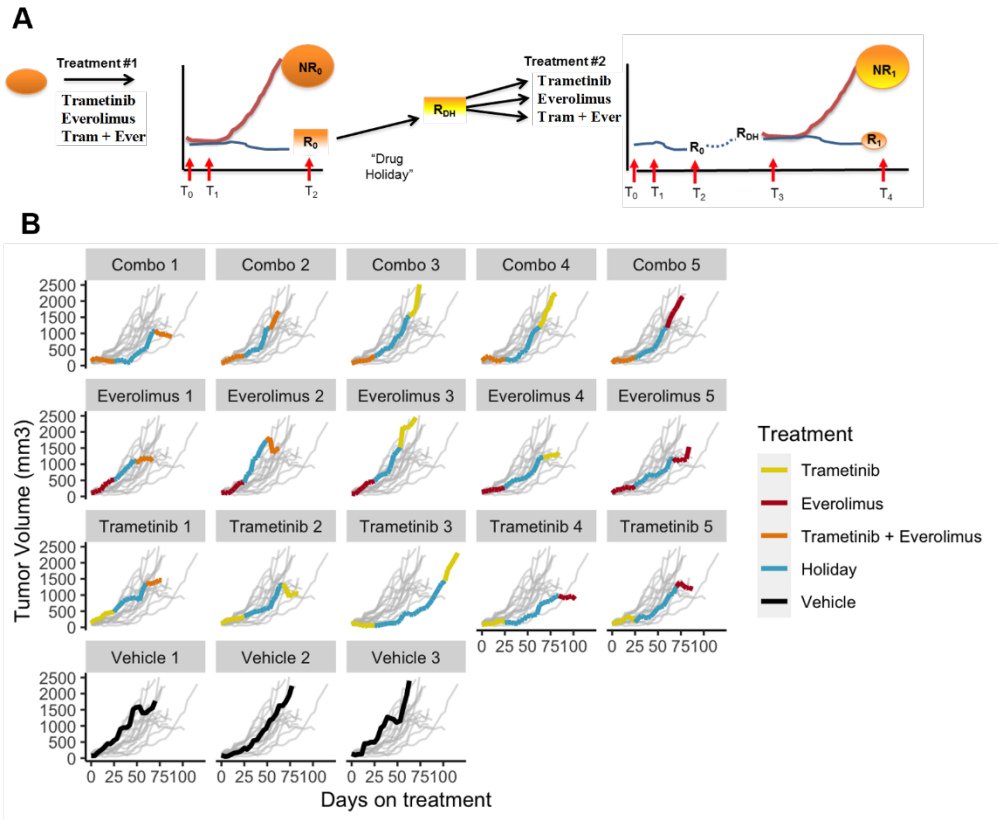

Figure 4: Modeling resistance to MEK and AKT inhibition in MPNSTs
A) Tumorgrafts are monitored until they reach 150 mm^3 (T_0), at which point they are randomized and placed on treatment (T_1). Tumors continue to be monitored for 15 days while they are on treatment (T_2), or until they reach euthanasia criteria at 2500 mm^3 (NR_o) and tumors collected. Tumors that reach the end of 15 days of treatment without reaching euthanasia criteria (R_o) are placed on a drug holiday and monitored twice weekly until they reach 1500 mm^3 (R_{DH}). At this point, the tumors are biopsied and randomized to either a new treatment or their previous treatment (T_3). Tumorgrafts are monitored until they reach euthanasia criteria (NR_1) or the end of the 15 days of treatment (T_4). B) Individual growth curves of MSTA-440-2 PDX lines treated with 1 mg/kg trametinib, 5 mg/kg everolimus, or a combination. Each treatment is represented by the corresponding color (as represented in legend) and plotted against all the individual tumor growth curves (gray).

continued to grow through the drug holiday. However, only one tumor, which switched treatment to trametinib alone, did not respond to secondary treatment. Other tumors, whether placed on the combination, everolimus alone, or even another tumor placed on trametinib alone appeared to respond if only for a short period to treatment. Mice treated initially with trametinib alone had a similar growth pattern to the everolimus treated mice, however there was a delayed response in tumor growth after treatment cessation. Likewise, one tumor that was placed on trametinib for the secondary treatment did not respond, while other treatments, including another trametinib treated tumor, responded initially to secondary treatment.

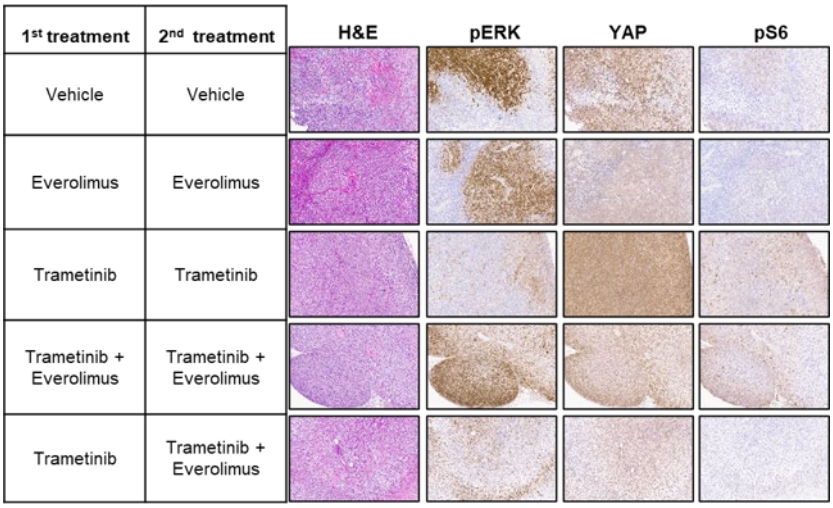

Figure 5: YAP activation present in treatment-resistant MPNST PDX tumors. Immunostaining of tumors from MPNST PDX shown in Figure 4 with pERK, YAP, and pS6.

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 reveals 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 5). This leads to the idea of a potential switch occurring between ERK and YAP signaling that could be behaving as a node of resistance in these tumors.

Current/Future Studies: For Aim 2, we are continuing to evaluate treatment resistance in additional MPNST PDX tumorgrafts. In addition, we are performing the statistical analysis to identify tumor responses in initial treatment vs. drug holiday vs. secondary treatment. The results from these studies have identified YAP signaling in resistant tumors, we are evaluating YAP-RAS signaling in both our in vitro and in vivo models.

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

MPNST variants can be identified with standard short read sequencing approaches, but due to the size of the NF1 gene and the complexity of MPNST structural alterations, a more reliable method is required to verify LOH events in NF1, RTK copy number variation, clonal heterogeneity, and the impact of haplotype context on somatic mutation function. To interrogate Nf1 isoform expression it is essential to utilize several sequencing methods because of the extreme length of the *NF1* genes (60 exons and > 300 kb of genomic DNA). Standard short-read sequencing approaches have provided important but incomplete details regarding the *NF1* genome and RNA sequence variation. Even though short-read sequencing is commonly used for transcript and isoform analysis, reads that span exon–exon junctions (and are used to identify distinct isoforms) can map indistinctly when a junction is shared between isoforms. This issue can complicate isoform analysis. Long-read cDNA methods can generate full-length isoform reads that substantially reduce these issues and improve differential isoform expression analysis. Although long-read sequencing has several advantages for isoform analysis, there are distinct limitations, as well, including lower throughput of long transcripts and higher error rates. To interrogate the range of *NF1* isoforms present in normal and malignant cells, we proposed to combine both long-read nanopore RNA sequencing technology and short-read RNA sequencing methods. For these experiments, we isolated RNA from WT control cells.

Optimization of long-read sequencing of *Nf1* transcripts

We developed RNA-probes that were used to pull down target sequences in *Nf1* in order to enrich for larger (>500 bp) transcript fragments. This hybridization method required large amounts of RNA template (>500 ng). For the RNA isolation, we isolated high molecular weight (HMW) RNA from $Nf1^{WT}$ and $Nf1^{mut}$ epithelial cells and tumor cells. Using *Nf1* targeted probes, we were able to pulldown *Nf1* RNA transcript fragments. In two separate runs on the Nanopore MinION sequencer, our analysis of the read length revealed that the majority of sequencing

reads were shorter than desired and ranged between 1-3Kb (Figure 6). Read length in Nanopore sequencing can be limited by the ability to deliver very HMW DNA to the pore as efficiently as shorter reads. This is a known challenge in the long-read sequencing field and an issue that several labs are working to optimize (Gordon Research Conference: Post-Transcriptional Gene Regulation, July 2022). The potential optimization methods that we will use to overcome these issues are described below.

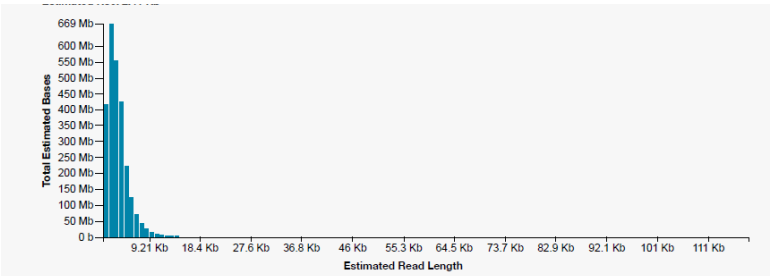

Figure 6: Sequencing read length on Nanopore MinION platform. The estimated read length of all sequences in a single run of combined samples.

Current/Future Studies: In the next year, we will continue to optimize our long-read sequencing using the following methods. 1) Use ReadFish to select target regions – using this software algorithm we can analyze the signal after a DNA molecule enters a pore to determine whether that molecule lies within a specified region of interest. If it does, the pore continues to sequence the molecule; if not, the DNA molecule is ejected from the pore. In this manner, we can focus on *Nf1* transcripts 2) We will utilize both polyA probes and *Nf1* probes during the targeted pulldown step. Since longer polyA tail length are more readily translated than shorter read tails, we will preferentially pulldown and sequence *Nf1* transcripts that are likely to be transcribed. When we have completed the optimization on control tissues, we will start sequencing MPNST tissues.

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, who is currently working on this study has presented her work at the AACR Sarcoma Meeting (May 2022) and will be presenting at the Fourth RAS Initiative Symposium 2022 (October 2022).

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 finalizing two manuscripts for publication in Dec 2022 and April 2023.

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

Our plans for each Aim are discussed above with each aim.

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.

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:	2.9 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:	3.5 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	1.7 calendar months or 14% 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*

10. References

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7. Kermorgant, S., Zicha, D. & Parker, P.J. PKC controls HGF-dependent c-Met traffic, signalling and cell migration. *Embo j* **23**, 3721-3734 (2004).