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14. ABSTRACT

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder that affects ~1/3500 newborns with a germline mutation in tumor suppressor gene NF1. A life-threatening complication of having NF1 is the development of an aggressive and highly metastatic malignant peripheral nerve sheath tumor (MPNST) at an earlier age compared to the general population. Currently, there are no effective treatments for MPNST other than complete surgical resection with wide margins. Patients with NF1 develop MPNSTs through the malignant transformation that is accompanied by a genetic model: 1) About 50% of NF1 patients exhibit plexiform neurofibromas, which are caused by the loss-of- function in NF1 and the associated hyper activated Ras signaling pathway, 2) Atypical neurofibromas arise from PNs and they exhibit loss of CDKN2A, and 3) approximately 13% of NF1 patients develop MPNSTs, in which recurrent mutations in SUZ12 and/or EED, two key components of the polycomb repressive complex 2 (PRC2), were identified, leading to loss of tri-methylation of histone H3 lysine 27 (H3K27me3) in these tumors. PRC2 and its product H3K27me3 are critical epigenetic modifiers contributing to the maintenance of transcriptional repression of essential genes for normal cellular function. To explore the epigenetic changes following PRC2 loss, I profiled the epigenetic landscapes in MPNSTs and found that histone marker of super enhancers, acetylated-H3K27 (H3K27ac), was gained as H3K27me3 was lost, indicating the potential impact of super enhancer-defined core transcriptional regulatory circuitry (CRC) in driving MPNST tumorigenesis.

15. SUBJECT TERMS
Cancer, Prostate Cancer, Oncology

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1. INTRODUCTION:

Malignant peripheral nerve sheath tumor (MPNST) is an aggressive soft tissue sarcoma that lacks effective treatment. It predominantly arises from patients with neurofibromatosis type 1 (NF1), which is the most common genetic disorder that affects 1/3000 newborns in the US. Recurrent genetic alterations in the core component genes of polycomb repressive complex 2 (PRC2), SUZ12 or EED, were identified in 60-90% of all MPNST. Since PRC2 possesses the activity of methyltransferase, its loss leads to the global loss of trimethylation of histone H3 at lysine 27 (H3K27me3) in human MPNST samples. Follow up studies comparing PRC2-deficient and PRC2-wildtype MPSNT demonstrated that the loss of PRC2 causes gain of H3K27ac, activation of Ras signaling, as well as reduction of immune related signals. However, the mechanistic explanations of these observations associated with PRC2 loss remain unveiled. In this current study, I proposed to characterize the transcriptional functions of PRC2-regulated transcription factors in human MPNST cells with the ultimate goal of targeting the epigenetic vulnerabilities of PRC2-deficient MPNST using re-purposed small molecule inhibitors.

2. KEYWORDS: Neurofibromatosis type 1, malignant peripheral nerve sheath tumor, polycomb repressive complex 2, transcription factors, super enhancer, transcriptional regulation, drug screen

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Training-Specific Tasks:
Major Task 1: Training and educational development in neurofibromatosis research.
Proposed Milestone(s): Presentation of project data at NF Conference.
Achieved (yes/no, date): Yes. Project data was presented at weekly joint lab meetings, two invited presentations, and two international conferences. More details are listed in "6. Products".

Research-Specific Tasks:
Specific Aims:
Aim 1: To experimentally validate the interconnected transcriptional regulatory loop that is defined by PRRX2 in PRC2-null MPNST cells.
Major Task 1: Cell culture, treatment, and sequencing assays.
Proposed Milestone(s): all ChIP-DNA, ATAC-DNA, and RNA submitted for sequencing to the CCR Genomic Core.
Achieved (yes/no, date): Yes. All ChIP-DNA, ATAC-DNA and RNA samples were submitted for sequencing to the CCR Sequencing Facility by October 9, 2020.
Major Task 2: Data analysis and integration.
Proposed Milestone(s): experimental validation of the PRC2-null MPNST specific core

transcriptional regulatory circuitry defined by PRRX2.
Achieved (yes/no, date): Yes. All data analysis of ChIP-seq, ATAC-seq and RNA-seq were completed and integrated in the submitted manuscript by June 1, 2021.

Aim 2: To perform a large-scale drug screen for effective chemical compounds that mimic the effect of PRC2 restoration in PRC2-null MPNST cells.

Major Task 3: Build the reporter cell lines and perform the initial screen.
Proposed Milestone(s): completing of the initial screen with fewer compounds that specifically inhibit the super enhancer activity of PRRX2.
Achieved (yes/no, date): No. Explanations of progress made are listed below.

Major Task 4: Perform the secondary screen.
Proposed Milestone(s): identification of final candidate drugs that exhibit dose-dependent inhibition of super enhancer activity and MPNST cell proliferation.
Achieved (yes/no, date): No.

Major Task 5: RNA-seq on final candidate drugs and data analysis.
Proposed Milestone(s): identification of drugs that transcriptionally mimic the effect of PRC2 restoration in PRC2-null MPNST cells; publication of 1-2 peer-reviewed papers
Achieved (yes/no, date): No.

What was accomplished under these goals?

Research-Specific Tasks:

Aim 1: To experimentally validate the interconnected transcriptional regulatory loop that is defined by PRRX2 in PRC2-null MPNST cells.

Loss of PRC2 is prevalently detected in 60-90% of all MPNSTs and it is the last step of genetic alterations during the malignant transformation when benign plexiform neurofibromas develop into malignant peripheral nerve sheath tumors (MPNST). PRC2 is a multi-subunit epigenetic protein complex that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27me3) and is required for proper maintenance of transcriptional repression of key genes during development and cell lineage determination. In MPNST, the loss of PRC2 results in a diverse set of consequences, including hyperactivated Ras signaling, increased expression of WNT pathway genes, and downregulation of genes involved in immune surveillance. Additionally, gain of acetylated H3K27 (H3K27ac) accompanying the loss of H3K27me3 has been observed in PRC2-deficient MPNST. Mechanistic understanding of how the loss of PRC2 results in these diverse, but specific consequences remain unresolved. In this project, I proposed to restore a functional PRC2 in three PRC2-deficient MPNST cell lines and profile the transcriptomic and epigenetic changes accompanied the dynamic reassembly of PRC2.
As stated in the Project Narrative, I screened a panel of 10 human MPNST cell lines and used Western blotting of H3K27me3 to identify 3 PRC2-deficient cell lines (T265, ST88-14, and PT2002). I restored a functional PRC2 and decoration of H3K27me3 in these cells by using the doxycycline (Dox) inducible expression vector of human *SUZ12* open reading frame (Figure 1). The cells were treated with 0.5 μg/mL Dox for 5 consecutive days to achieve the SUZ12 expression level similar to a PRC2-competent cell line.

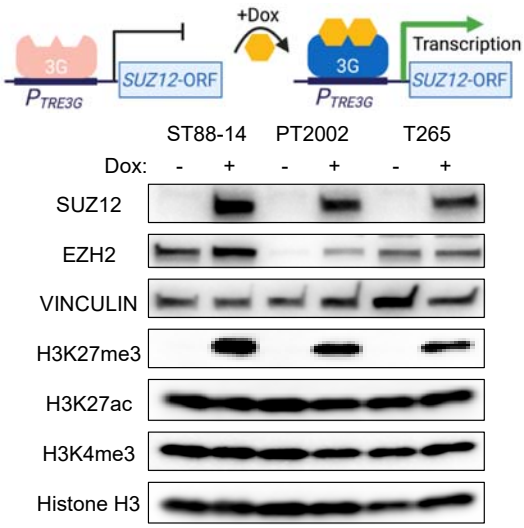


Figure 1. Doxycycline (Dox) induced re-expression of SUZ12 in ST88-14, PT2002 and T265 cells restored trimethylated histone H3 lysine 27 (H3K27me3) shown by western blotting.

I used RNA sequencing to profile the transcriptomic alterations in these cells when a functional PRC2 was restored and found that a total of 1683 and 1357 genes were significantly downregulated and upregulated, respectively, upon the restoration of a functional PRC2 (p_{adj}<0.05). The dominant transcriptional alteration (Figure 2) was the downregulation of genes involved in axonogenesis (p_{adj}=8.92E-19), embryonic organ (p_{adj}=6.51E-10) and development regionalization (p_{adj}=6.01E-12). Strikingly, multiple PRC2-regulated forkhead (FOX) or homeobox (HOX) transcription factors (TF) that are critical for cell fate specification were transcriptionally downregulated by PRC2, including *FOXC1* (log2FC=-1.30, p_{adj}=2.81E-05), *FOXA2* (log2FC=-2.44, p_{adj}=1.82E-06), *HOXB8* (log2FC=-3.11, p_{adj}=6.08E-11) and *PRRX2* (log2FC=1.29, p_{adj}=1.33E-07).

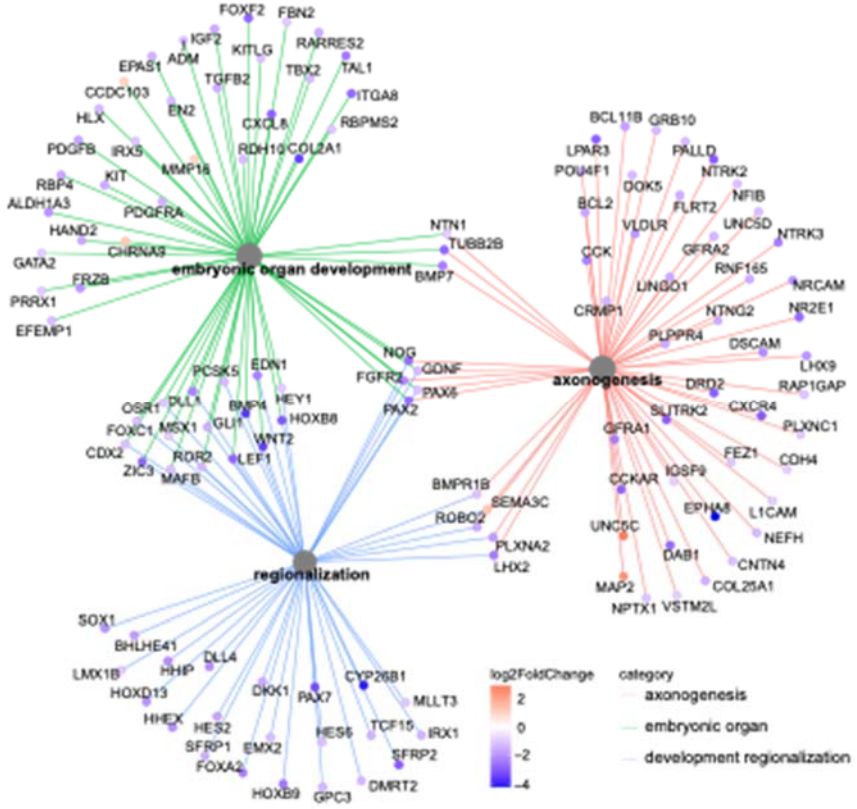


Figure 2. Network plot demonstrating the connection of 3 significantly enriched pathways of the differentially expressed genes (DEG) caused by PRC2 restoration. DEG were derived from a combined analysis of the RNAseq results obtained from all three PRC2-null MPNST cell lines, comparing samples with induced expression of SUZ12 to controls.

To characterize the effects of PRC2 restoration on global gain in H3K27me3 on the genome, we profiled its distribution using chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq) in the inducible cell system. Genome wide, while there was no H3K27me3 signal in PRC2-deficient cells, upon reconstitution of PRC2, we identified 81549, 48275, and 33897 significant H3K27me3 peaks in ST88-14, PT2002 and T265 cells, respectively. Notably, there were 6134 peaks common to all three cell lines (Figure 3).

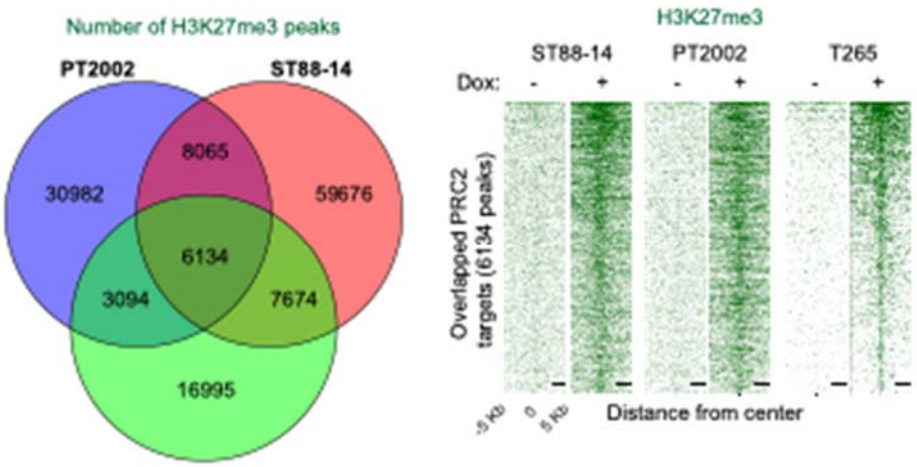


Figure 3. Left panel: Venn diagram showing the number of overlapped H3K27me3 peaks in three MPNST cell lines upon PRC2 restoration. Right panel: PRC2 restoration induced by SUZ12 re-expression drives the genome wide gain of H3K27me3 occupancy demonstrated by overlapped ChIP peaks in ST88-14, PT2002 and T265 cells.

Interestingly, although PRRX2 was found to be transcriptionally regulated by PRC2 (its RNA level was reduced and its super enhancer (SE) was diminished by PRC2 restoration), it did not gain H3K27me3 at the promoter (Figure 4). It is possible that PRRX2 is transcriptionally regulated by another direct target gene of PRC2. In order to identify and further characterize a direct PRC2 targeted transcription factor that is essential for the survival of MPNST cells, I performed a customized small interfering RNA (siRNA) screen. I went back to the originally identified TFs that were both highly expressed in human MPNST samples and also associated with an autoregulated SE (defined if its own binding motif was identified within its assigned SE) in the three inducible model systems. In total, we compiled a list of 43 MPNST specific SE-driven TFs that were present in any of the three cell lines and performed a siRNA screen using a customized library. Three unique siRNAs targeting each candidate gene were selected to ensure coverage across the transcript. Results of the siRNA screen indicated that 18/43 (42%) of the TFs were critical for T265 (medium growth reduction rate > 30%,) and 23/43 (53%) of the TFs were essential for ST88-14 cells (Figure 5). Common essential TFs included *SNAI2*, which demonstrated a 75% growth reduction in both cell lines. Interestingly in the inducible model system, six TFs: *FOXC1*, *HOXB8, MSX2, FOXD2, FOXD1 and HOXD13* demonstrated both loss of H3K27ac and corresponding gain of H3K27me3 indicating that the decreased expression of these TFs was a direct result of PRC2 restoration (Figure 6). Two PRC2 direct target TFs, *FOXC1* and *HOXB8* were selected for further interrogation because of their association with significant growth reduction in both cell lines. We confirmed the growth rate reduction with individual siRNA-mediated knockdown of these two TFs and observed that the growth rate was dependent on the protein dose for *FOXC1* and *HOXB8*. CRISPR-Cas9

knockout of *FOXC1* or *HOXB8* in T265 cells resulted in a significant reduction of colony formation when *FOXC1* was knocked out, and a trend of decreased colony formation in the *HOXB8* knockdown cells (Figure 7).

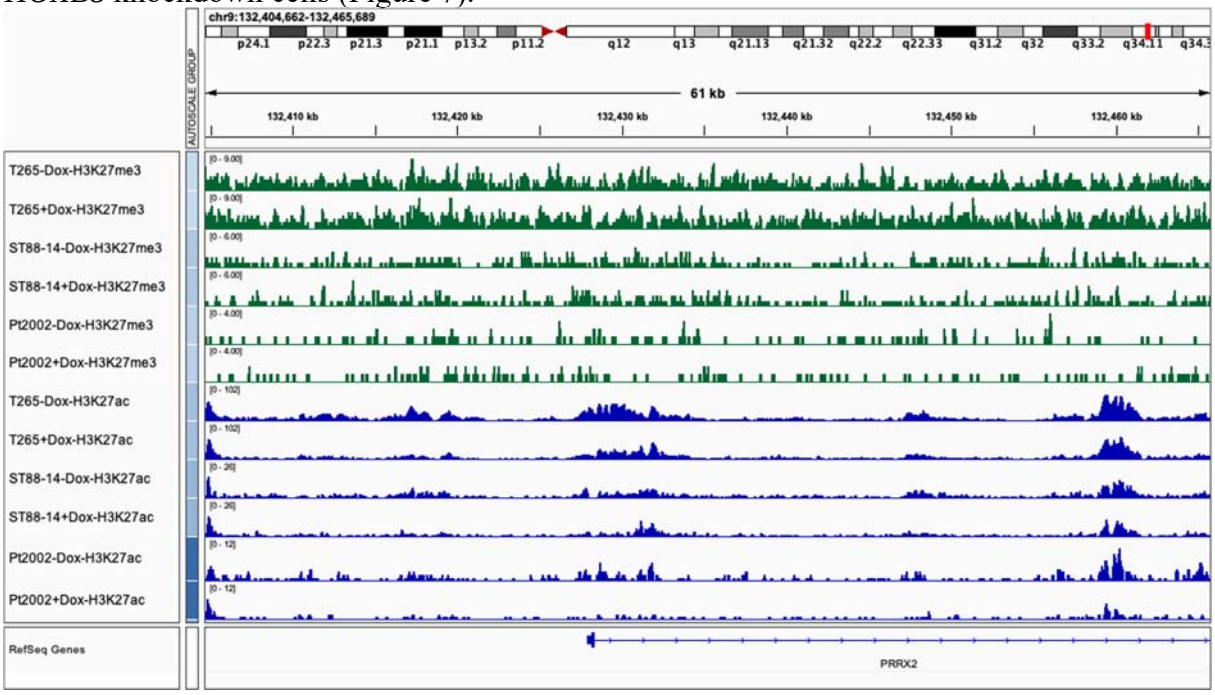


Figure 4. Genome browser of ChIP-seq result shows that PRRX2 is not a direct PRC2 target in MPNST cells.

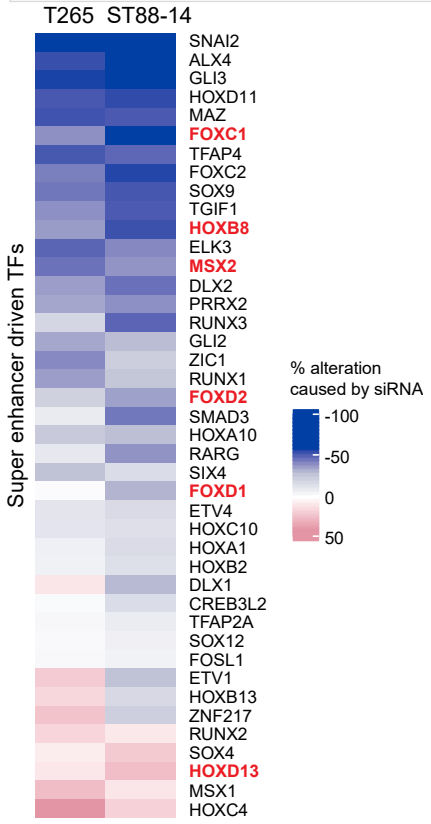
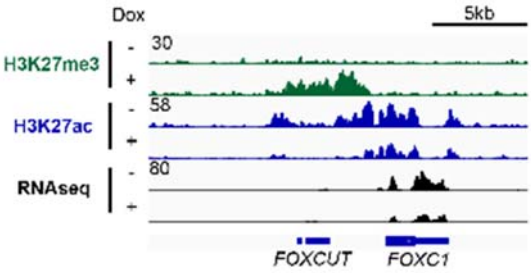


Figure 5. Heatmap showing the median percentage of reduction in proliferation when a candidate core TF was knocked down using three distinct siRNAs, in comparison to the control siRNA. Data shown in the order of average median reduction from the highest to the lowest in two cell lines. Data summarizes two replicates per siRNA, per cell line. Direct PRC2 targets are highlighted in red.



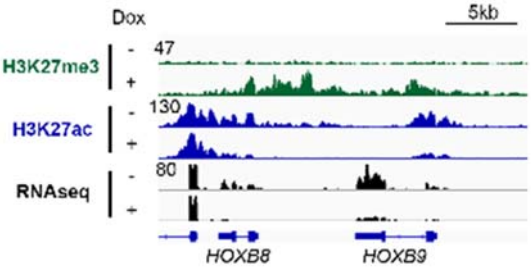


Figure 6. Example IGV track around the *FOXC1* (upper panel) and *HOXB8* (lower panel) loci demonstrate the gain of H3K27me3, loss of SE and transcriptional reduction accompanied with the Dox-induced PRC2 restoration in T265 cells.

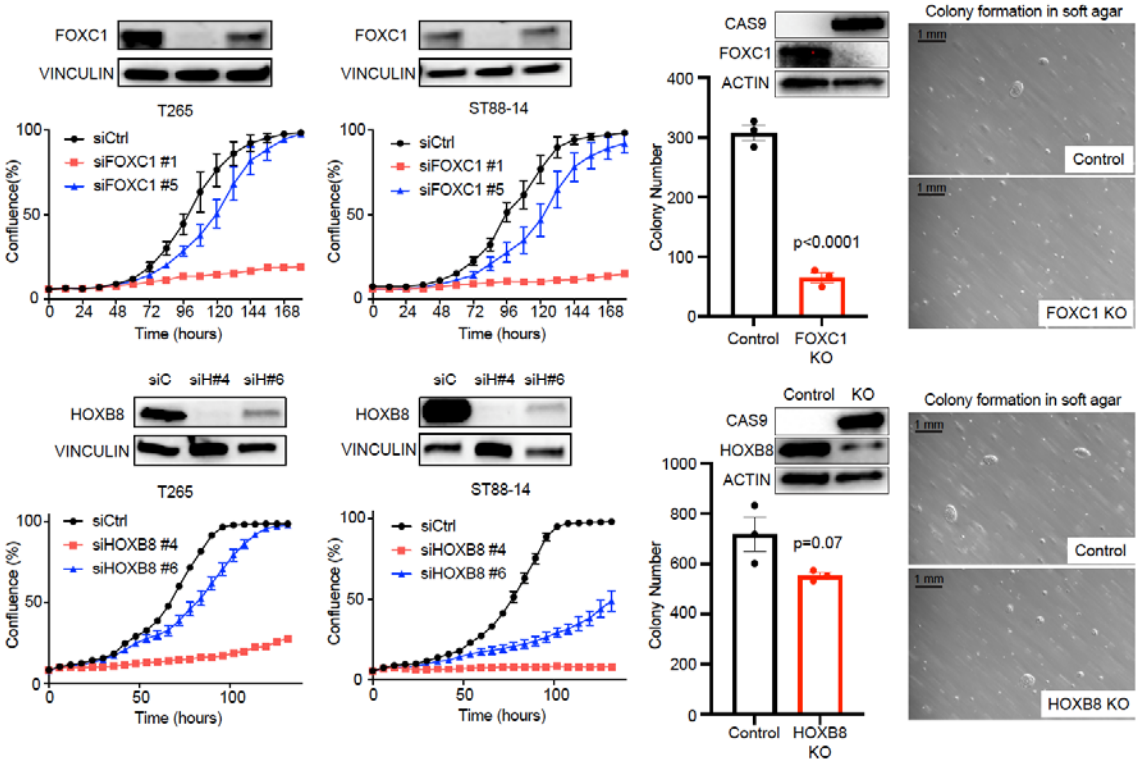


Figure 7. Upper panel: Cell growth assay using individual siRNAs targeting FOXC1 in T265 (first panel) and ST88-14 (second panel). Western blotting of FOXC1 72 hours after the transfection using siControl (siC), siFOXC1-#1 (siF#1) and siFOXC1-#5 (siF#5). Quantification (third panel) and representative picture (forth panel) of colony formation when FOXC1 was knocked-out in T265 cells by CRISPR-Cas9. Lower panel: Cell growth assay using individual siRNAs targeting HOXB8 in T265 (first panel) and ST88-14 (second panel). Western blotting of HOXB8 72 hours after the transfection using siC, siHOXB8-#4 (siH#4) and siHOXB8-#6 (siH#6). Quantification (third panel) and representative picture (forth panel) of colony formation when HOXB8 was knocked-out in T265 cells by CRISPR-Cas9. Cell growth graph plots the monitored cell confluency against elapsed time after the transfection. The average of five replicates is presented. Mean ± SEM. Scale bar: 1 mm.

Given the direct PRC2 regulation and significant effect on MPNST cell viability, FOXC1 was selected for further mechanistic characterization. FOXC1 has been reported to bind individual promoters and activate target genes, but its binding has not previously been assessed genome-wide. FOXC1 specific ChIP-seq was performed to determine its genome binding in T265 cells with or without the Dox-induced PRC2 restoration. A total of 11613 peaks (p=10E-5), associated with 4869 genes, were identified in PRC2-deficient T265 cells. Among these peaks, only 2.8% (281 peaks) were present at gene promoters and 50.1% (5821 peaks) of the discovered peaks overlapped with H3K27ac-based active enhancers as defined by ROSE (Figure 8).

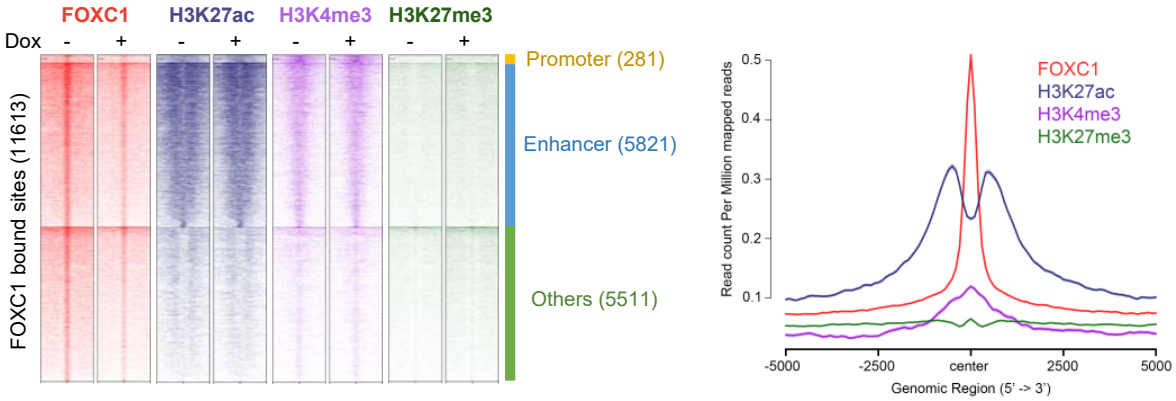


Figure 8. Left panel: Heatmap of ChIP-seq signal intensity of FOXC1, H3K27ac, H3K4me3 and H3K27me3 peaks in T265 without (-Dox) or with (+Dox) a functional PRC2 restored. ChIP-seq signals are calculated over 11613 FOXC1 peaks that were segmented to three categories: promoter (281 peaks, within 1000 bp from the transcription start site), enhancer (5821 peaks, overlapped with enhancers determined by ROSE using H3K27ac ChIP-seq result) and others (5511 peaks, not at promoters or do not overlap with enhancers). Right panel: Composite plot of FOXC1, H3K27ac, H3K4me3 and H3K27me3 ChIP-seq signal intensity over 11613 FOXC1 binding sites, showing that FOXC1 peaks overlap with H3K27ac signals.

Notably, FOXC1 binding at highly expressed genes overlapped with SEs decorated by long stretches of H3K27ac peaks near the gene body, indicating that it may play an important role in regulating these genes in MPNST cells (Figure 9).

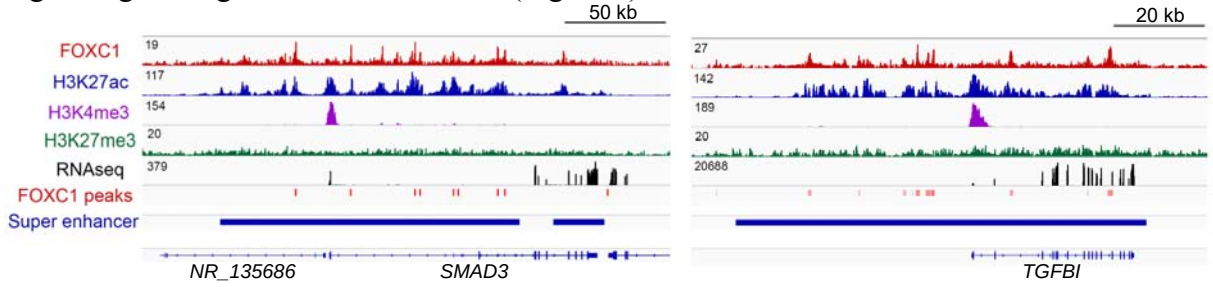


Figure 9. Example IGV tracks around the *SMAD3* locus (left panel) and *TGFBI* locus (right panel), showing multiple FOXC1 peaks decorate these super enhancers.

To evaluate the regulatory elements associated with FOXC1 binding to the genome, we performed motif analysis of the binding sites and enriched pathway analysis of the associated targeted genes. FOXC1-bound regions were significantly enriched in four classes of motifs: 1. basic region leucine zipper (bZIP) domain-containing TFs including AP1 family TFs; 2. winged-helix domain-containing FOX TFs; 3. runt homology domain-containing TFs, including RUNX1, RUNX2, and RUNX3; and 4. homeobox domain-containing HOX TFs

(Figure 10). Notably, like FOXC1, these TFs were highly expressed in MPNSTs, had a SE associated with the TF gene, and this SE was frequently bound by clusters of FOXC1 peaks, as shown for *SMAD3* (Figure 9) and *RUNX2* (data not shown) loci. Importantly, interrogation of the motifs of these genes' H3K27ac peaks predicted that they bound each other's SE (Figure 11). Therefore, we hypothesized that FOXC1, induced by PRC2 loss, plays a critical role in MPNST oncogenesis by establishing a core TF regulatory circuit. Additionally, we hypothesized that PRC2-regulated FOXC1 forms a core transcriptional regulatory circuitry together with other essential, highly expressed, SE-driven TFs that may or may not be regulated by PRC2. These TFs are critical for regulating the oncogenic transcriptional program of MPNST cells by driving a lineage-specific phenotype.

FOXC1 HOMER *de novo* motif

Rank	Motif	*P*-value	Best match
1		1e-1599	FOSL1, FOSL2, JUNB, ATF3, AP1
2		1e-586	FOXO3/4/6, FOXI1, FOXK1/2, FOXD1, FOXP1
3		1e-485	RUNX1, RUNX2, RUNX3
4		1e-182	HOXD11, HOXA11, HOXA9, HOXA13

Figure 10. A table presenting the HOMER motif analysis results using FOXC1 ChIP-seq peaks. The top 4 *de novo* motif categories and the best matched TFs representing that motif are shown.

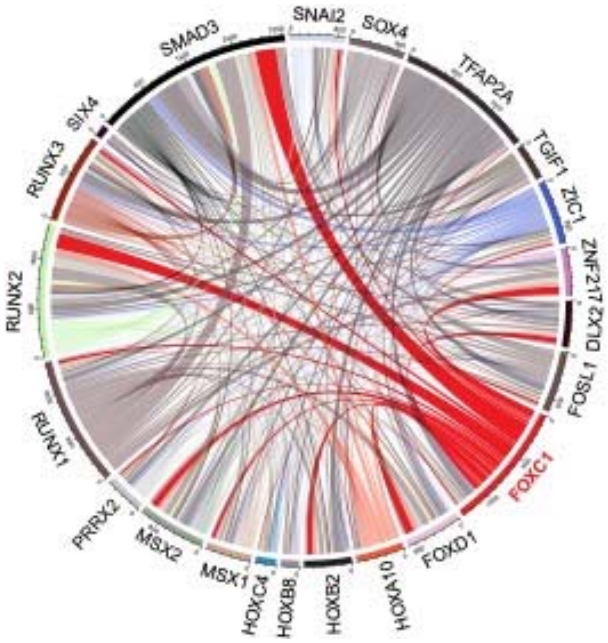


Figure 11. Chord diagram showing the number of predicted motifs of MPNST-specific SE-driven TFs found in their own and each other's SE. The width of a colored line coming out of a TF represents the number of predicted motifs of that particular TF that can be found in the SE of the other TF, which is the destination of that colored line.

Finally, to characterize the transcriptional regulation of FOXC1, I created MPNST cell lines in which FOXC1 was knocked out through CRISPR-Cas9 mediated genome editing and profiled the transcriptome of these cells to be compared to the parental cells. This result was integrated with the ChIP-seq result of FOXC1 to identify the direct FOXC1 target genes. RNAseq of CRISPR-Cas9 knockout of *FOXC1* identified that 45% (195/436) of the downregulated, and 24% (92/377) of the upregulated genes had a corresponding FOXC1 ChIP-seq peak (Figure 12).

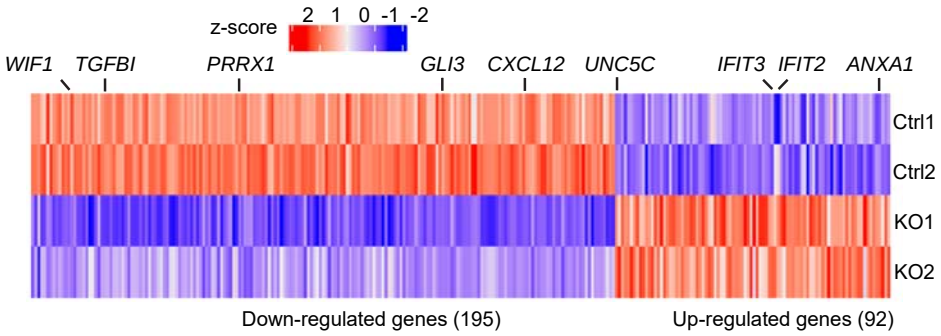


Figure 12. Heatmap shows the transcriptional alterations of direct FOXC1 targeted genes upon CRISPR-Cas9 mediated knockout of FOXC1 in T265 cells.

In summary, these findings indicated that FOXC1 coordinates a core transcriptional regulatory circuit at SEs of highly expressed genes and drives MPNST oncogenesis by activating critical pathways, including Ras (Figure 13).

Figure 13. Schematic illustration of FOXC1's key role in regulating the oncogenic program of MPNST.

Aim 2: To perform a large-scale drug screen for effective chemical compounds that mimic the effect of PRC2 restoration in PRC2-null MPNST cells.

In the Project Narrative, I originally proposed to engineer a reporter cell line to indicate the SE activity of PRRX2 and use this reporter system in drug screen for effective chemical compounds that specifically inhibit the SE activity. To test if the SE activity of PRRX2 is sufficient to indicate the anti-tumor activity when its SE is transcriptionally silenced, I first used the CRISPR-inhibition (CRISPRi) system that accurately achieves transcriptional inhibition through KRAB-mediated gene silencing. I created the T265 and ST88-14 CRISPRi systems by transfecting the parental cell lines using lenti-virus of dCas9-KRAB plasmid, followed by antibiotic selection using G418. When transfected with guide RNAs (gRNAs) targeting a specific genomic locus, the targeted region will be transcriptionally silenced by the recruited epigenetic machinery through increased H3K9me3 and reduced H3K27ac. I used gRNAs targeting the SE of PRRX2 in these two CRISPRi systems and found that transcription of PRRX2 was reduced in these cells (Figure 14a) when its SE was targeted by KRAB protein, accompanied by reduction in H3K27ac signal intensity and increase of H3K9me3 signals (Figure 14b). However, targeting the SE of PRRX2 did not affect the survival of these cells (Figure 14c). These results indicate that we need a better readout for the proposed drug screen. I therefore am currently performing the drug screen using the engineered cell lines and am looking for drugs that selectively inhibit the growth of these cells without the Dox-inducible PRC2 restoration.

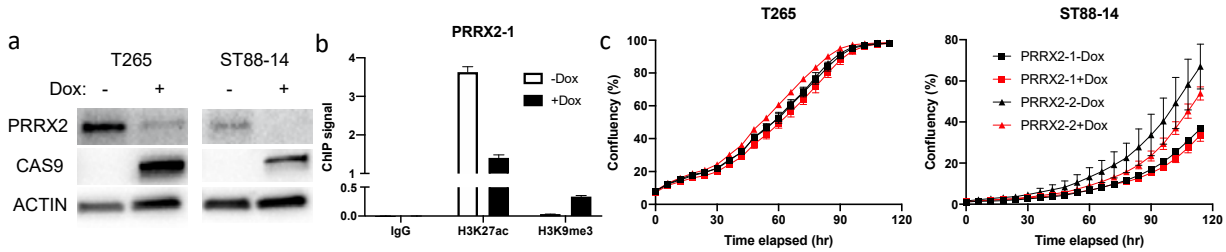


Figure 14. Transcriptional silencing of PRRX2 by targeting its SE. a. Western blotting shows that protein of PRRX2 was diminished in the Dox-indicible CRISPRi systems when the cells were treated with Dox. b. ChIP-qPCR results show that CRISPRi system worked when H3K27ac was reduced and H3K9me3 was increased by the KRAB protein. c. Plot of cell confluency shows that transcriptional silencing of PRRX2 by targeting its SE did not inhibit the survival of MPNST cells.

What opportunities for training and professional development did the project provide? **How were the results disseminated to communities of interest?**

Training-Specific Tasks:

Major Task 1: Training and educational development in neurofibromatosis research. The project has provided me the following opportunities for training and professional development.

Training:

- Bi-weekly one-on-one meeting with my primary mentor, Dr. Jack Shern.
- One-on-one meeting with my co-mentor, Dr. Brigitte Widemann, every 2-3 months.
- Training course, titled "Python Programming for Scientists Series*", offered by the NIH was taken in April 2020. This course includes 10 half-day workshops covering varies topics of using Python as a programming language in Biomedical research. *This course was taken as a substitute for the proposed workshop Programming for Biomedical Researchers.
- Training course, titled "Statistical Analysis of Research Data*", offered by NCI was taken from January to February 2021. *This course was taken as a substitute for the proposed course Statistics for Biomedical Scientists.

Professional development:

- Sallie Rosen Kaplan (SRK) and Diversity Career Development Program Fellowship* from 2019-2020. * The SRK Fellowship is a highly competitive, unpaid, annual, one-year program that provides additional mentoring opportunities, networking, seminars, and workshops to help prepare NCI's female postdoctoral fellows for the competitive nature of the job market and help us to transition to independent research careers. The program

addresses the fact that, based on recent observational, longitudinal, and intervention studies, women in science are significantly more likely to leave the science field earlier than men, specifically at the transition from a mentored scientist to an independent investigator. The highlight of this program was a 30-week course entitled "Career Building for Women in Science", which includes two day-long workshops. Following each day-long session, we had the opportunity to network with the facilitator in a 2-hour, bi-weekly meeting (for 14 weeks). The SRK Fellowship also included mentoring opportunities with successful women scientists as a secondary mentor as well as seminars on relevant topics such as networking, managing people, time management, and how to self-promote. This rewarding program has equipped me with the necessary tools that I need in the current challenging job market, as a female scientist from a different culture background.

- AACR annual conference in 2020* and 2021. *I was awarded the Women in Cancer Research Award in 2020.
- NF annual research conference in 2020 and 2021.
- Invited to present at the Transcription Scientific Interest Group webinar for my award-winning abstract of the annual Fellows Award for Research Excellence in October 2020.
- Organized the inaugural "Fellow-invited speaker seminar" at the Pediatric Oncology Branch of NCI. This event included a presentation given by a fellow-invited speaker and a virtual "coffee-hour" with the invited speaker.
- Participated several of the virtual "coffee hour" with renowned researchers to discuss research questions and career development options.

How were the results disseminated to communities of interest?

- Presentation at multiple occasion including AACR annual conference, NF annual conference, NIH-wide Transcription Scientific Interest Group, and Pediatric Oncology Branch at NCI.
- Submission of one manuscript to scientific journal.
- Participation at the NF hackathon in 2020. Since NF1-associated tumors originate from Schwann cells and MPNST exhibit a de-differentiated phenotype, my collaborator and I analyzed publicly available single-cell RNA sequencing data obtained from developing Schwann cells at different developmental stages. We integrated the analysis and built a normal Schwann cell development trajectory to be used to compare the malignant cells from MPNST to normal Schwann cells. Results of this project is part of the submitted manuscript and available through our Github page. We believe that this result is of great interest to the research community of neurofibromatosis.
- Support the summer internship. I have been mentoring summer interns every year since I came to the NCI in 2016. This year, all summer internships were help virtually. I lectured on "Data analysis in R" to a group of 18 summer interns with diverse background and gave a scientific presentation on "Single-cell Sequencing in Cancer Research". Besides these group activities, I also mentored a student on her project studying the protein interactomes of the core transcription factor, FOXC1, which we identified to be a key vulnerability of

MPNST caused by PRC2 loss. These efforts have provided learning opportunities to the next generation researcher who are interested in learning more of the NF research that we are doing in the lab.

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

Training-Specific Plans:
I will continue the bi-weekly meetings with my primary mentor and bi-monthly meetings with my co-mentor to discuss research progress and career development in the next reporting period. To present my research to the NF community, I have submitted an abstract that was selected to be presented at the 1st Neurofibroma Young Investigator Forum (NFYIF) in September 2021. I also submitted an abstract to present this project at the Connective Tissue Oncology Society annual conference, which will be held in November 2021. I also plan to present my work at weekly joint research meetings at the Pediatric Oncology Branch at NCI. Once the new schedule of courses offered by NIH is available, I will register for the course "The Art of Drug Design and Discovery" in the fall semester of 2021.

Research-Specific Plans:
For Aim 1, all proposed research have been completed and integrated into the submitted manuscript (DoD funding was acknowledged). Additionally, I am interested in characterizing the inter-connected transcriptional regulatory circuitry formed by groups of core transcription factors in MPNST, so I submitted two more research grants to propose the use of single-cell CRISPR sequencing in MPNST cells.

For Aim 2, since targeting the super enhancer of PRRX2 was not able to affect the viability of MPNST cells, I will not continue to use the reporter system in the proposed drug screen. Instead, I have initiated the drug screen using the engineered human MPNST cell lines, in which a functional PRC2 is restored when the cells are treated with doxycycline. I am currently screening the drugs that have differential sensitivity in cell viability in PRC2-restored cells when compared to the PRC2-deficient counterparts. To target the key vulnerability of MPNST cells, the essential transcription factor FOXC1, I used combinatorial treatment of small molecule MEK inhibitor and bromodomain inhibitor JQ1. The combination of these two drugs is in clinical development and they synergistically killed MPNST cells *in vitro*. Interestingly, they significantly reduced the protein level of FOXC1 when used together to treat MPNST cells.

4. IMPACT:

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

Polycomb repressive complex 2 (PRC2) is a well-known transcriptional regulator of normal cellular developmental processes, and genetic alteration of PRC2 is a common feature of multiple cancer types. These alterations have been associated with diverse phenotypes including metastatic disease, therapeutic failure, and lack of response to immune therapy; however, the mechanistic details underpinning these findings is relatively unexplored. As proposed in Aim 1 which later was completed and integrated into a recently submitted manuscript, we investigate the oncogenic mechanism of the loss of PRC2 using malignant peripheral nerve sheath tumor (MPNST), a histology where PRC2 loss is a common final step in oncogenic transformation. While a rare tumor, MPNST provides a unique cellular model to broadly understand the consequences of PRC2 loss, given the nearly universal incidence of genetic loss of structural components of a PRC2 and corresponding lack of H3K27me3 in these tumor cells. The experimental work partially funded by this grant includes a detailed transcriptomic and epigenetic profiling of MPNST model systems, as well as the first use of single cell sequencing of patient clinical samples.

As detailed in the submitted manuscript, we used engineered tumor cells to dynamically reassemble a competent PRC2 and document the biochemical, transcriptomic, and epigenetic consequences of the restoration of the complex. These experiments allowed us to define PRC2-specific target genes, and discover that 1) only a small portion (12%) of PRC2-targets are transcriptionally de-repressed as a result of PRC2 loss, while the majority of genes remain silenced or unaltered regardless of the status of PRC2; 2) transcriptionally regulated PRC2-targets are frequently bivalent chromatin domains, which are co-decorated by H3K27me3 and H3K4me3; 3) signal intensity of its mutually exclusive histone mark, H3K27ac, is significantly reduced as a result of genome-wide redistribution of H3K27me3.

Leveraging our unique cellular model systems, we comprehensively characterized the enhancer landscape in tumor cells to investigate the impact of PRC2 on H3K27ac and the associated active enhancers. This work led to the discovery that PRC2 loss results in the upregulation of a transcriptional circuit that remodels the chromatin landscape of the tumor cells. Interestingly, we uncovered that PRC2 loss specifically upregulates several transcription factors, including FOXC1. We provide detailed characterization of this master transcription factor's genomic distribution and document it as a core vulnerability of the tumor cell. As an unanticipated finding, our work revealed that loss of PRC2 indirectly represses interferon signaling and antigen presentation as a downstream consequence of hyperactivated Ras signaling. Treatment with a small molecule MEK inhibitor reversed this immune suppressive transcriptional profile and these findings have potential therapeutic implications for ongoing and planned clinical trials.

Finally, we extended our findings in the model systems, by performing single-cell RNA sequencing (scRNAseq) and single-cell whole genome sequencing (scCNV) of clinical tumor samples. This approach enabled us to compare the PRC2-deficient MPNST cells with the benign Schwann cells (PRC2-intact) from the same patient and led to the discovery that the transcriptional program enforced by PRC2 loss is highly correlated with gene signatures of a de-differentiated mesenchymal stem cell. Our dataset and novel bioinformatic approach

enabled us to build a normal neural crest stem cell developmental trajectory and overlay the malignant cells onto this "map". Remarkably, using our clinical samples, we discovered a specific mesenchymal stem cell transcriptional profile that is enforced by the abnormal activation of PRC2-regulated transcription factors, including FOXC1. To our knowledge, this is the first report to leverage single-cell transcriptomic analysis to advance the understanding of the de-differentiated cellular phenotype in a tumor cell.

PRC2 regulation is a key regulator of normal developmental processes and its alteration is a common feature of multiple cancers. Our study provides detailed mechanistic understanding of PRC2-mediated oncogenesis and its unique relationship to fundamental cellular developmental processes. These findings and the associated datasets generated, once published, will significantly impact our understanding of the transcriptional regulations through polycomb-mediated epigenetic mechnisms.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

During the reporting period, I presented my work partially funded by this grant to various audience, including our branch, NIH, as well as at international conferences. These presentations have positive impact on improving public knowledge of the disease we study. More and more general audience are aware of this disease.
I participated in the NF hackathon during the reporting period. This event was to encourage researchers use publicly available data to solve problems that patients, physicians or researcher

of NF may face. My collaborator and I used publicly available single-cell sequencing data to build a normal Schwann cell developmental trajectory. We demonstrated that the de-differentiated phenotype that malignant NF1 tumors exhibit trapped them in a specific stage of normal development. We have made our script and discovery available so it may improve public knowledge and skills.
My newly hired postbac fellow and I are preparing to write an article about NF1 to be published at "Frontiers for Young Minds". This journal is an open-access scientific journal written by scientists and reviewed by a board of kids and teens. Since NF and NF related diseases are pediatric disease, our goal is to raise awareness of this disease among young audience. We hope to improve the social environment for patients with NF by introducing this disease to the young audience of this journal.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.
Training-Specific Tasks:
Due to the many changes caused by the COVID-19 pandemic, a lot of proposed course and conferences were cancelled or re-formatted (to become virtual). Additionally, the lab was completely shut down from March to July in 2020. I therefore made a few changes to the proposed Training-Specific Tasks to utilize the "stay-at-home" time during the shut-down. As reported in "3. Accomplishments", I took the online course, titled "Python Programming for Scientists Series" to replace the originally proposed workshop "Programming for Biomedical Researchers" in April 2020. I also took the online course "Statistical Analysis of Research Data" as a substitute for the originally proposed course "Statistics for Biomedical Scientists". The contents of these two courses overlapped with the originally proposed courses.

Research-Specific Tasks:
We have no changes in objectives and scope to report. Currently, we are working on the drug screen proposed in Aim 2. We aim to look for drugs that exhibit differential sensitivity in reducing the cell viability in PRC2-restored cells compared to PRC2-deficient cells. Instead of using the originally proposed reporter system that specifically reports the super enhancer activity of PRRX2, which I demonstrated earlier that has no effect in cell viability, I will perform the initial drug screen using the engineered PRC2-restored MPSNT cells with or without the presence of doxycycline. I will look for the drugs that demonstrate selective sensitivity in PRC2-restored cells than the PRC2-deficient cells. As initially proposed, I will continue the secondary drug screen with fewer drugs to validate their efficacy in reducing cell viability before moving into the final stage of the screen, which is to perform transcriptomic profiling of treated cells to find the ones that mimic the effect of PRC2 restoration in MPNST cells.

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

Due to the delay in staff hiring, lab closure last year, and slow ramping up in research activity, we faced delay in starting the proposed drug screen. We are currently at nearly full capacity of carrying out the proposed projects. I do not foresee any further delay unless there is another round of lab shutdown due to the recent spreading of delta variant of the COVID-19 virus.

Changes that had a significant impact on expenditures

We have been dealing with the consequences caused by the COVID-19 pandemic since March, 2020. I had postponed the start date of this project to August 1st, 2020, due to the forced closure of our institute from March to July. However, even though we were able to open the lab in July 2020, our institute and lab were required to operate at 25-50% capacity. I therefore was not able to start the hiring process until March 2021, when I posted the job advertisement about the proposed Postbac fellow on the NCI website. Ms. Hilda Jafarah, a recent graduate from Pittsburg University, was selected and hired with a start date of July 6 2021. She is currently being trained by me to work on the proposed study, including the extension of characterizing the MPNST-specific core transcriptional regulatory circuitry and the drug screen that targets the epigenetic vulnerability of MPNST.

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:
Publications, conference papers, and presentations

Publications:

- Xiyuan Zhang, Hannah E. Lou, Vishaka Gopalan, Zhihui Liu, Haiyan Lei, Paige Jones, Carly M. Sayers, Marielle E. Yohe, Prashant Chittiboina, Brigitte C. Widemann, Carol J. Thiele, Michael C. Kelly, Sridhar Hannenhalli, Jack F. Shern. Loss of PRC2 enforces a mesenchymal neural crest stem cell phenotype in NF1-deficient cancer through activation of core transcription factors. *Nature Cell Biology*. [*submitted*] acknowledgement of federal support (yes)

Invited presentations:

- Loss of Polycomb Repressive Complex 2 Amplifies a FOXC1-Dependent Transcriptional Regulatory Circuitry in Malignant Peripheral Nerve Sheath Tumors. October 2020, Transcription SIG Webinar, NIH, Bethesda, MD, USA
- Single-cell resolution of the diverse epigenetic and transcriptomic reprogramming of NF1 deficient cells through the loss of PRC2. April 2021, Pediatric Oncology Branch Research Seminar, NCI, Bethesda, MD, USA

Conference papers:

- **Xiyuan Zhang**, Haiyan Lei, Cecilia Tibery, Zachary Rae, Allison Ruchinskas, Michael Kelly, Markku Miettnen, Brigitte Widemann, and Jack Shern. Single-cell RNA Sequencing Identified Increased Schwann Cell Population as a Marker of Atypical Neurofibromatous Neoplasms with Uncertain Biologic Potential, 2020. Children's Tumor Foundation Annual NF Conference, virtual. *[Poster presentation]*
- **Xiyuan Zhang**, Hannah Lou, Haiyan Lei, Zhihui Liu, Marielle Yohe, Carol Thiele, Brigitte Widemann, and Jack Shern. Loss of polycomb repressive complex 2 activates HOXB8 and remodels super enhancers in malignant peripheral nerve sheath tumors, 2020. AACR Annual Meeting, virtual. *[Poster presentation]*

Journal publications:

- Xiyuan Zhang, Hannah E. Lou, Vishaka Gopalan, Zhihui Liu, Haiyan Lei, Paige Jones, Carly M. Sayers, Marielle E. Yohe, Prashant Chittiboina, Brigitte C. Widemann, Carol J. Thiele, Michael C. Kelly, Sridhar Hannenhalli, Jack F. Shern. Loss of PRC2 enforces a mesenchymal neural crest stem cell phenotype in NF1-deficient cancer through activation of core transcription factors. *Nature Cell Biology*. [*submitted*] 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):
https://github.com/vishakagopalan/nf_hackathon
This website contains the Jupyter notebook and accompanying code for the 2020 NF

hackathon. The PI and her collaborator, Dr. Vishaka Gopalan, participated in the 2020 NF hackathon. We performed integrated analysis of publicly available single-cell RNA sequencing data and built a normal Schwann cell developmental trajectory. This website made sure that the product of this collaboration is available to be shared with the research community.

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:

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Name:	Xiyuan Zhang
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ORCID: 0000-0001-9949-5258
Nearest person month worked:	12
Contribution to Project:	Conducted the proposed research, prepared manuscript #1 that was submitted, and supervised the Postbaccalaureat Research Fellow funded by this grant.
Funding Support:	NIH Intramural Research Funding and this grant.

Name:	Jack Shern
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	0000-0001-5579-7625
Nearest person month worked:	1

Contribution to Project:	Supervised the research conducted by the PI.
Funding Support:	NIH Intramural Research Funding

Name:	Brigitte Widemann
Project Role:	Co-mentor
Researcher Identifier (e.g. ORCID ID):	0000-0002-9198-7175
Nearest person month worked:	1
Contribution to Project:	Supervised the research conducted by the PI.
Funding Support:	NIH Intramural Research Funding

Name:	Hilda Jafarah
Project Role:	Postbaccalaureat Research Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0003-0957-0408
Nearest person month worked:	1
Contribution to Project:	Conducted the proposed experiments.
Funding Support:	This grant.

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?
Organization Name: Vishaka Gopalan
Location of Organization: Bethesda, MD
Partner's contribution to the project: Collaboration
I collaborated with Dr. Vishaka Gopolan from the Laboratory of Cancer Data Science. We teamed up and participated in the 2020 NF Hackathon.

8. SPECIAL REPORTING REQUIREMENTS:

QUAD CHARTS:

9. APPENDICES:

None