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PRINCIPAL INVESTIGATOR: Dr. Xiyuan Zhang

CONTRACTING ORGANIZATION: The Geneva Foundation, Tacoma, WA

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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 Oncology, Cancer, Prostate Cancer					
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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 MPNST 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, five invited presentations, two international conferences, and one NF Young Investigators' Forum. 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 published work (Zhang *et al.* Cell Reports)

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): Yes. RNAseq was performed in cells treated with a new class of HDACi, combinatorial treatment by bromodomain inhibitor (JQ1) and MEK inhibitor. The data analysis of the RNAseq was completed in May 2022. One peer-reviewed paper was published in Sept. 2022. Another peer-reviewed paper was published in May 2023.

What was accomplished under these goals?

The proposed aim1 including all major tasks in aim1 have been completed. Through RNAseq, ChIP-seq, and ATAC-seq, I have profiled the interconnected transcriptional loop formed by PRC2 and its regulated transcription factors (TFs). Results of these studies are now published at Cell Reports (see appendix for more details). Additionally, data/dataset from this study were deposited to GEO and released to the public.

To target the TFs that were activated by PRC2-loss, I proposed to perform a large-scale drug screen for small molecules that mimic the effect of PRC2 restoration (effective in inhibiting cell proliferation and reducing levels of PRC2-regulated TFs). Originally I proposed to screen for drugs that effectively target the transcriptional activity of PRC2-specific TFs. However, preliminary result shows that reduction of transcription through targeting the super enhancer of PRRX2 had no effect on cell viability. Additionally, we found out that Dr. Kyle Williams, a postdoctoral fellow in Dr. David Largaespada's group, has performed a drug screen to target PRC2 loss. They reported at

conferences that histone deacetylase inhibitor (HDACi) exhibits efficacy in treating MPNST and it synergized with MEK inhibitor in cell culture and animal model of MPNST. Along this line, I have tested a new class of HDACi, which is a dual inhibitor targeting both HDAC and AKT (protein kinase B, also activated by the activated Ras signaling through the loss of NF1). The dual HDACi, CUDC907, has been tested in multiple MPNST cell lines to be effective in inhibiting the cell proliferation (Figure 1). More importantly, CUDC907 was effective in reducing the protein levels of the PRC2-targeted TFs (Figure 2).

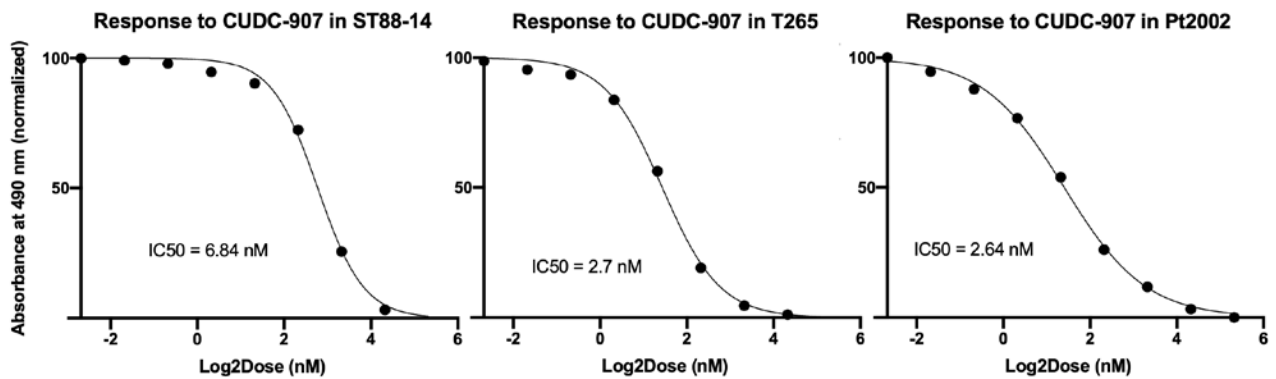


Figure 1. CUDC907 exhibits efficacy in treating MPNST cells.

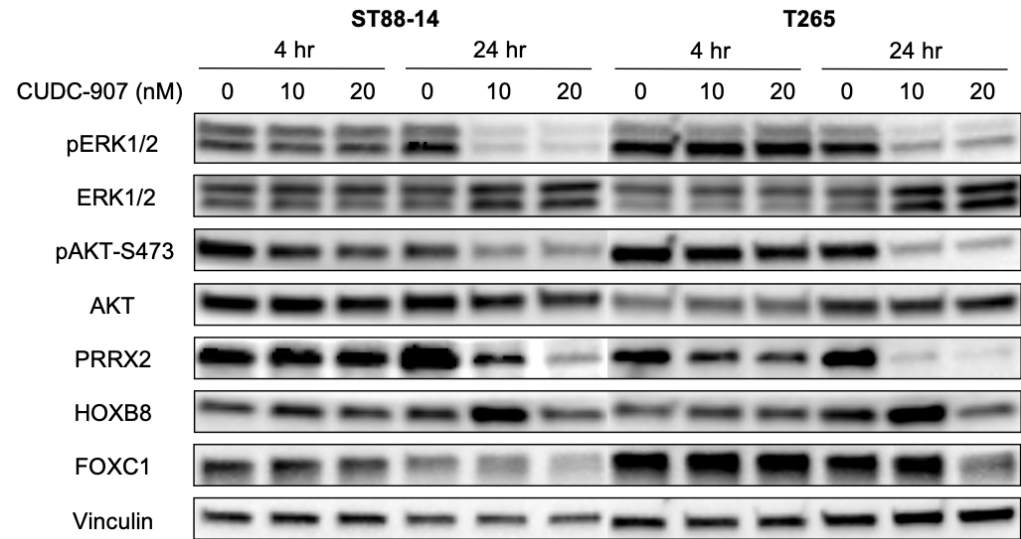


Figure 2. CUDC907 treatment reduced protein levels of PRC2-targeted TFs in MPNST cell lines.

Additionally, I performed transcriptomic profiling using RNA sequencing (RNAseq)in MPNST cells treated with CUDC-907 for different lengths of time. As proposed in the grant, I treated the cells for 4 and 24 hours and harvested cells for RNA extraction, RNAseq, and analyzed the data using a standard pipeline as well customized bioinformatic tools. As shown in **Figure 3**, CUDC-907 treated cells demonstrated a dominant transcriptional activation response (left panel) and results of the geneset enrichment analysis confirmed the transcriptional effect of HDAC inhibitor in these cells. Importantly, key super enhancer-driven transcription factors were down-regulated by CUDC-907 treatment in a dose- and time-dependent manner (Figure 4).

T265 cells treated by 20 nM CUDC-907 for 24 hr

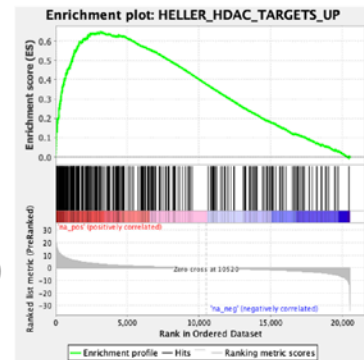
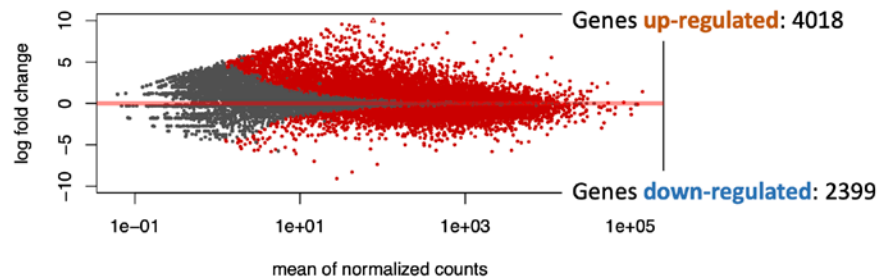


Figure 3. MPNST cells treated with dual HDAC and AKT inhibitor demonstrated more transcriptional activation than repression.

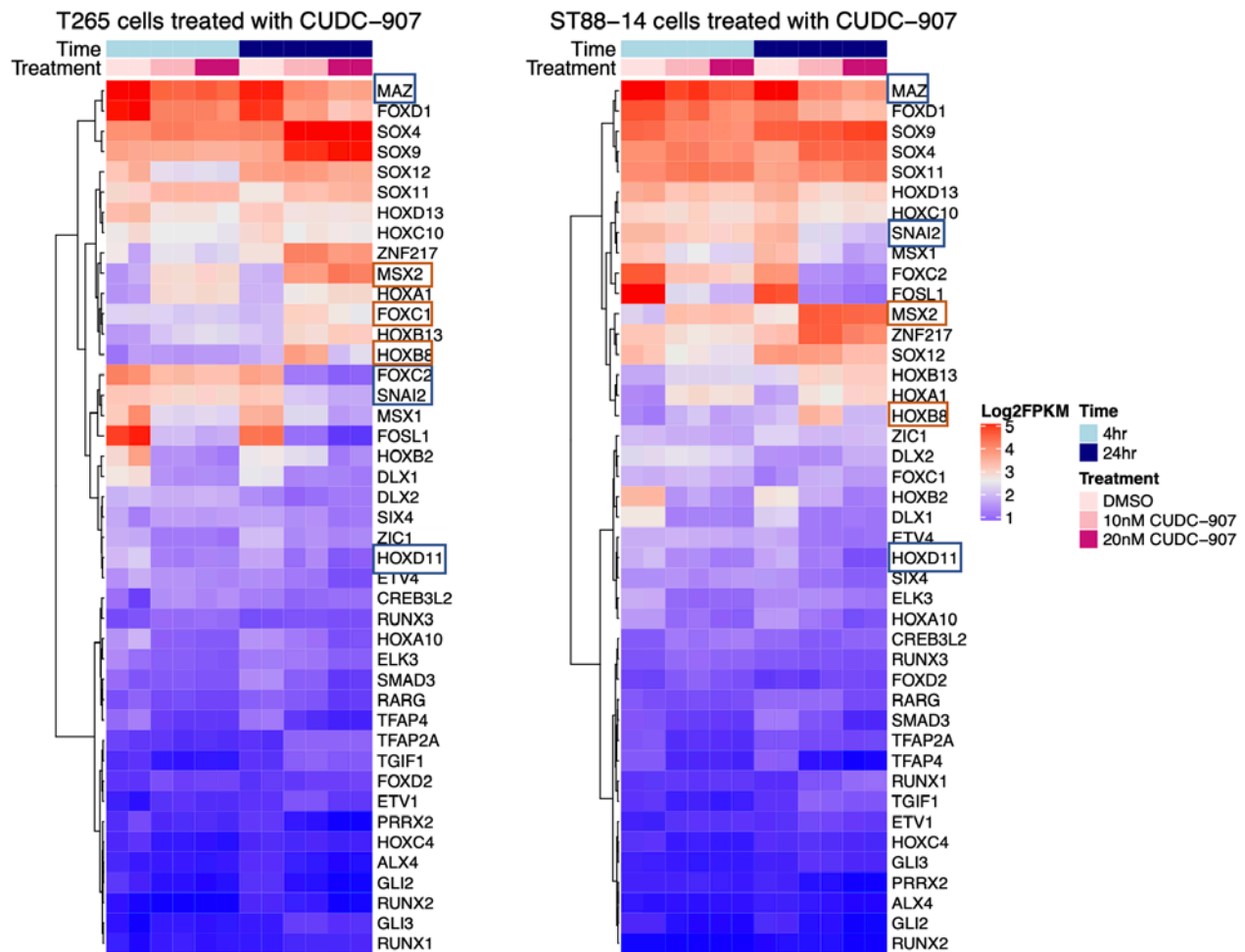


Figure 4. CUDC-907 treatment reduced the RNA levels of PRC2-targeted TFs in a dose- and time-dependent manner.

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

PI

During the reporting period, Dr. Zhang presented results of this study three times, including at annual neurofibromatosis young investigators' forum, pediatric oncology branch research seminar, and Children's Tumor Foundation NF annual conference (International conference).

Postbac fellow

Miss Hilda Jafarah was hired as a postbac fellow to work on the proposed projects in this study. She has been working under the mentorship of Dr. Zhang and Dr. Jack Shern in the Pediatric Oncology Branch. She attended annual meeting of AACR, BMES and research roundup of the POB.

How were the results disseminated to communities of interest?

- Publication and released data/database deposited

I have published one peer-reviewed journal articles (Zhang *et al.* Cell Reports; Zhang *et al.* Star Protocols) and the related data generated was deposited to GEO and released to the public upon the publication of this work. More details can be found in the Appendix.

- Presentations at international conferences, research seminar, and research forum

I was invited to present this work 3 times in the last reporting period. Details could be found in "6. Products".

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

Dr. Zhang will continue to be provided with training opportunities towards her career goals. I will wrap up the findings of the drug study and prepare another manuscript to be submitted for peer review in 2024.

4. IMPACT:

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

This study has enabled us to identify highly potent, selectively inhibitory drugs that are effective in treating MPNSTs by targeting the PRC2-regulated super enhancer-driven transcription. The epigenetic and transcriptomic profiling data and methods of data analysis were shared upon publication of this study to benefit researchers of the NF research community. Additionally, support from this study has benefited both the PI and the postbac to meet training and educational expectations. Because of the support, Miss Hilda Jafarah has successfully completed her training at the NIH and will begin her graduate study at the University of Pennsylvania.

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?

Nothing to Report.

5. CHANGES/PROBLEMS:

Nothing to Report.

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

.

There was a delay in completing the aims outlined in the proposal that prompted a request for a no cost extension which was awarded on 19 May 2023. With the extension, the timeline is now back on track for timely completion.

Changes that had a significant impact on expenditures

Nothing to Report.

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

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

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

Journal publications.

Published: **Xiyuan Zhang**, Hannah E. Lou, Vishaka Gopalan, Zhihui Liu, Hilda M. Jafarah, Haiyan Lei, Paige Jones, Carly M. Sayers, Marielle E. Yohe, Prashant Chittiboina, Brigitte C. Widemann, Carol J. Thiele, Michael C. Kelly, Sridhar Hannenhalli and Jack F. Shern. Single-cell sequencing reveals activation of core transcription factors in PRC2-deficient malignant peripheral nerve sheath tumor. **Cell Reports**. 2022 Sep 20;40(12):111363. doi: 10.1016/j.celrep.2022.111363.
acknowledgement of federal support: Yes

Published: **Xiyuan Zhang***, Vishaka Gopalan, Neeraja Syed, Sridhar Hannenhalli and Jack F. Shern. Protocol for using single-cell sequencing to study the heterogeneity of NF1 nerve sheath tumors from clinical biospecimens. **Star Protocols**. 2023 May 10. doi: 10.1016/j.xpro.2023.102297. **co-correspondence*
acknowledgement of federal support: Yes

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



Other publications, conference papers and presentations.

Xiyuan Zhang (invited speaker) 2022. Single-cell sequencing reveals transcriptomic diversity that facilitates the malignant transformation of NF1 nerve sheath tumors. *Pediatric Oncology Branch Research Seminar*, NCI, Bethesda, MD, USA

Xiyuan Zhang (invited speaker and winner of presentation competition) 2022. Single-cell sequencing reveals transcriptomic diversity that facilitates the malignant transformation of NF1 nerve sheath tumors. *2nd Annual Neurofibromatosis Young Investigators' Forum*, Houston, TX, USA

Xiyuan Zhang (invited platform speaker) 2023. Single-cell sequencing reveals transcriptomic diversity that facilitates the malignant transformation of NF1 nerve sheath tumors. *Children's Tumor Foundation (CTF) NF Conference*, Scottsdale, AZ, USA

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

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

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

Nothing to Report.

- **Other Products**

Along with the publication of this work (Zhang *et al.* Cell Reports), we released the related dataset that was deposited to GEO.

Data: Raw and processed next generation sequencing data generated for this study have been deposited in the NCBI GEO with the accession number GEO: GSE183309. This SuperSeries is composed of the following SubSeries: GSE183305, GSE183306, GSE183307, and GSE183308, containing related ATAC-seq, ChIP-seq, RNAseq, and single-cell RNAseq data, respectively.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xiyuan Zhang
Project Role:	PI
Researcher Identifier:	0000-0001-9949-5258
Nearest person month worked:	4
Contribution to Project:	Dr. Zhang has supervised and performed all of the proposed work in this study.
Funding Support:	NIH intramural research program and the Concept Award (DoD Rare Cancer Research Program).
Name:	Hilda Jafarah
Project Role:	Postbac fellow
Researcher Identifier:	N/A
Nearest person month worked:	12
Contribution to Project:	Miss Jafarah has performed work addressing the concerns that reviewers raised during the revision of the manuscript (Zhang <i>et al.</i> Cell Reports).
Funding Support:	NIH intramural research program.

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?

My work partially sponsored by this award was accepted as an oral presentation by the 2nd Annual Neurofibromatosis Young Investigators' Forum (NFYIF). NFYIF provided in-kind support for my travel to present my work in Houston.

Organization Name: Creative Educational Concepts (CEC)

Location of Organization: Kentucky

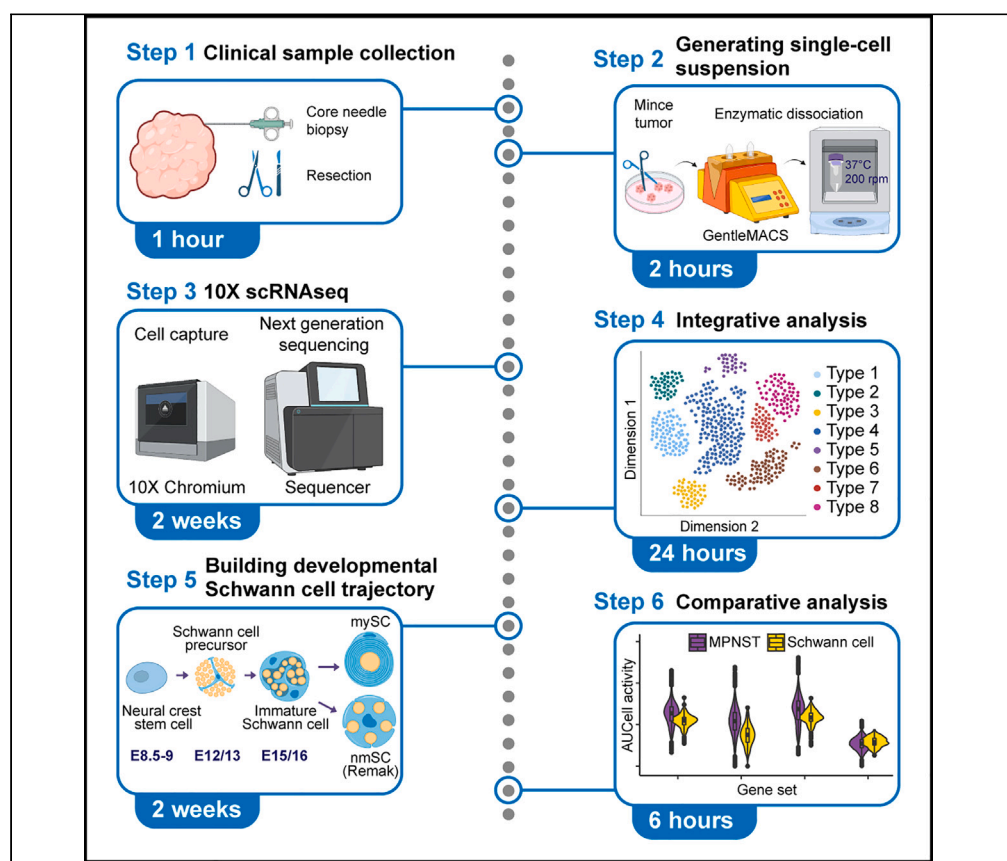
Partner's contribution to the project: In-kind support

8. APPENDICES:

Attached is a copy of the published journal articles (Zhang *et al.* Cell Reports; Zhang *et al.* Star Protocols)

Protocol

Protocol for using single-cell sequencing to study the heterogeneity of NF1 nerve sheath tumors from clinical biospecimens



Xiyuan Zhang,
Vishaka Gopalan,
Neeraja Syed,
Sridhar Hannenhalli,
Jack F. Shern

xiyuan.zhang@nih.gov
(X.Z.)
john.shern@nih.gov
(J.F.S.)

Highlights

Procedure for single-cell suspension preparation from patient NF1 nerve sheath tumors

Single-cell RNA sequencing using the 10x Chromium workflow

Integrative analysis of data collected from multiple patients to correct batch effect

Building a developmental Schwann cell trajectory for comparative analysis

Single-cell sequencing is a powerful technology to understand the heterogeneity of clinical biospecimens. Here, we present a protocol for obtaining single-cell suspension from neurofibromatosis type 1-associated nerve sheath tumors for transcriptomic profiling on the 10x platform. We describe steps for clinical sample collection, generation of single-cell suspension, and cell capture and sequencing. We then detail integrative analysis, developmental Schwann cell trajectory building using bioinformatic tools, and comparative analysis. This protocol can be adapted for single-cell sequencing using mouse nerve tumors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Zhang et al., STAR Protocols 4,
102297
June 16, 2023
<https://doi.org/10.1016/j.xpro.2023.102297>



Protocol

Protocol for using single-cell sequencing to study the heterogeneity of NF1 nerve sheath tumors from clinical biospecimens

Xiyuan Zhang,^{1,4,*} Vishaka Gopalan,² Neeraja Syed,³ Sridhar Hannenhalli,² and Jack F. Shern^{1,5,*}¹Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA²Cancer Data Science Laboratory, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA³Pediatric Oncology Branch Childhood Cancer Data Initiative, Frederick National Laboratory for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA⁴Technical contact⁵Lead contact*Correspondence: xiyuan.zhang@nih.gov (X.Z.), john.shern@nih.gov (J.F.S.)
<https://doi.org/10.1016/j.xpro.2023.102297>

SUMMARY

Single-cell sequencing is a powerful technology to understand the heterogeneity of clinical biospecimens. Here, we present a protocol for obtaining single-cell suspension from neurofibromatosis type 1-associated nerve sheath tumors for transcriptomic profiling on the 10x platform. We describe steps for clinical sample collection, generation of single-cell suspension, and cell capture and sequencing. We then detail methods for integrative analysis, developmental Schwann cell trajectory building using bioinformatic tools, and comparative analysis. This protocol can be adapted for single-cell sequencing using mouse nerve tumors.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022).¹

BEFORE YOU BEGIN

The protocol below describes the specific steps for single-cell RNA sequencing of a single-cell suspension obtained from fresh NF1-associated nerve sheath tumors. Transcriptomic profiling of all cell types on a single-cell level is a powerful tool to understand the intra-tumoral heterogeneity of these tumors. This protocol can be used on both fresh surgical specimens as well as patient-derived xenografts to profile the transcriptome on a single-cell level.²

Institutional permissions (if applicable)

Institutional permission for obtaining and studying clinical specimens should be obtained. Informed consent needs to be obtained from individual patients enrolled in a relevant clinical protocol. Informed consent was obtained on NCI IRB approved protocol (NCT01109394; IRB identifier 10C0086) for the tumor samples prospectively collected and included in this work. Samples are ordered and tracked through a clinical trial data management system. Upon receipt, the samples are assigned a unique barcode ID that is delinked from all identifying information and used for all downstream analysis.

Preparation of tumor dissociation media on the day before the surgery

⌚ Timing: 1 h



1. Prepare the DNase I solution as described in the [materials and equipment](#) section.
 - a. Prepare the 10× DNase I buffer.
 - b. Dilute the 10× DNase I buffer with Hanks' Balanced Salt Solution (HBSS) to 1× DNase I buffer.
 - c. Inject 20 mL of the 1× DNase I buffer to reconstitute the lyophilized DNase I so that the final concentration is 100 Kunitz units/mL.
 - d. Store the DNase I solution in 200 µL aliquots at −80°C. They are stable for a year.
2. Prepare the sterile tumor dissociation media as described in the [materials and equipment](#) section.
 - a. Weigh the dispase II and collagenase I to be dissolved in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin-glutamine.
 - b. Filter the enzyme cocktail with a 0.22 µm filter unit and store at 4°C.

Preparation on the day of surgery

⌚ Timing: 1 h

3. Warm up the sterile tumor dissociation media to 37°C.
4. Add DNase I to the sterile tumor dissociation media (100 µL per 10 mL).
5. Prepare the following items to be used in the laminar flow hood before heading to the procurement room to collect specimens.
 - a. One 10 mL syringe.
 - b. gentleMACS™ C-tube
 - c. Surgery scalpel and sterile tweezer.
 - d. 60 mm tissue culture dish.
 - e. 40 µm cell strainer.

⚠ **CRITICAL:** A minimum of 10 mL of complete tumor dissociation media is needed to process each specimen. Scale up the preparation of complete tumor dissociation media and tools for each additional specimen that is scheduled to be collected and processed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium	Thermo Fisher	Cat# 11995081
Fetal bovine serum	Bio-Techne	Cat# S11550
Penicillin-streptomycin-glutamine (100×)	Thermo Fisher	Cat# 10378016
Collagenase I	STEMCELL Technologies	Cat# 07416
Dispase II	MilliporeSigma	Cat# D4693-1G
DNase I	Invitrogen	Cat# 18047019
Bovine serum albumin	Sigma-Aldrich	Cat# A9418
ACK lysing buffer	Thermo Fisher	Cat# A1049201
AO/PI viability dye	Nexcelom	Cat# CS2-0106-5ML
Dulbecco's phosphate buffered saline	Sigma-Aldrich	Cat# D8537-500ML
Critical commercial assays		
Dead cell removal kit	Miltenyi Biotec	Cat# 130-090-101
EasySep Human CD56 Positive Selection Kit II	STEMCELL Technologies	Cat# 17855
Deposited data		
E9.5 neural crest cell single-cell sequencing data	Soldatov et al. ³	GSE129114
E12.5/E13.5 adrenal medulla cell single-cell sequencing data	Furlan et al. ⁴	GSE150150

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mature Schwann cell single-cell sequencing data	Wolbert et al. ⁵	GSE142541
Human NF1 nerve tumor single-cell sequencing data	Zhang et al. ¹	GSE183309
Software and algorithms		
Euler algorithm in TFBSTools	Tan and Lenhard ⁶	https://bioconductor.org/packages/release/bioc/html/TFBSTools.html
Cell Ranger v6.0.0	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest
Seurat v4.0	Butler et al. ⁷ ; Stuart et al. ⁸	https://satijalab.org/seurat/
inferCNV	inferCNV of the Trinity CTAT Project	https://github.com/broadinstitute/inferCNV
HOCOMOCO v11	Kulakovskiy et al. ⁹	https://hocomoco11.autosome.org
JASPER 2020	Fornes et al. ¹⁰	https://jaspar.genereg.net
SCENIC	Aibar et al. ¹¹	https://scenic.aertslab.org
Monocle3	Cao et al. ¹² ; Trapnell et al. ¹³	https://cole-trapnell-lab.github.io/monocle3/
Other		
0.22 µm filter unit	Millipore Sigma	Cat# SLGP033RS
10 mL Syringe	BD	Cat# 302995
MACS C-tube	Miltenyi Biotec	Cat# 130-096-334
Sterile surgery scalpel	N/A	N/A
40 µm cell strainer	Corning/Falcon	Cat# 352340
6 cm tissue culture dish	Corning	Cat# 353001
GentleMACS dissociator	Miltenyi Biotec	N/A
Cellometer Auto 2000	Nexcelom	N/A
Chromium Controller	10x Genomics	N/A
Illumina NextSeq machine	Illumina	N/A
Cell Counting Chamber	Nexcelom	Cat# SD100-514

MATERIALS AND EQUIPMENT

Tumor Dissociation Media		
Reagent	Final concentration	Amount
Dulbecco's modified eagle medium	N/A	45 mL
Fetal bovine serum	10%	5 mL
penicillin-streptomycin-glutamine (100×)	1×	500 µL
Dispase II	1 mg/mL	50 mg
Collagenase I	1 mg/mL	50 mg
DNase I solution (100 unit/mL)	1 Kunitz unit/mL	500 µL
Total	N/A	50 mL

Note: Prepare the Tumor Dissociation Media without adding the DNase I solution the day before the surgery. Store the sterile incomplete Tumor Dissociation Media at 4°C for a maximum of 24 h.

Note: Complete the preparation of the Tumor Dissociation Media by adding the DNase I solution immediately before the surgery.

10× DNase I buffer		
Reagent	Final concentration	Amount
Tris hydrochloride (1 M), pH 8.0	400 mM	4 mL
CaCl ₂ (1 M)	10 mM	100 µL
MgSO ₄ (1 M)	100 mM	1 mL
Hanks' Balanced Salt Solution	N/A	4.9 mL
Total	N/A	10 mL

Note: Store 10× DNase I buffer at 18°C–25°C for a maximum of 24 months.

DNase I solution		
Reagent	Final concentration	Amount
10× DNase I buffer	1×	2 mL
Hanks' Balanced Salt Solution	N/A	18 mL
DNase I (lyophilized)	100 Kunitz unit/mL	1 vial
Total	N/A	20 mL

Note: Store the DNase I solution in single-use aliquots for a maximum of 12 months.

Tissue dissociator

The gentleMACS™ from Miltenyi Biotec has been utilized in this protocol. This equipment is a bench-top instrument for the semi-automated dissociation of tissues into single-cell suspensions.

Alternatives: If a gentleMACS™ dissociator is not available in the laboratory, use of a 10 mL serological pipette to mix and agitate the minced tumor with Tumor Dissociation Media is acceptable.

STEP-BY-STEP METHOD DETAILS

Clinical sample collection

⌚ **Timing:** 1 h

This step describes the procedure of collecting clinical samples for single-cell sequencing.

1. Institutional approval and informed consent need to be in place before the usage of any clinical samples. In this protocol, the collected specimen can be obtained by surgical resection or core needle biopsy of the neurofibroma.
2. Biospecimen collection from patient neurofibroma.
 - a. Resected tumor may be obtained from a debulking surgery.
 - i. The surgically removed tumor comes from the operation room in a sterile container.
 - ii. Open the sterile container a laminar flow hood within the procurement suite.
 - iii. Apply pathology ink to the biospecimen to better visualize tumor margins.
 - iv. Measure the three-dimensional size of the inked specimen with a ruler and record the measurements.
 - v. Cut open the biospecimen lengthwise in the middle.
 - vi. Remove one 0.5 × 0.5 × 0.5 cm³ cube of tissue from one side and immediately place in DMEM in a 15 mL conical tube (Figure 1)
 - b. If the specimen is a core needle biopsy obtained for diagnostic purposes.
 - i. Obtain the ultrasound-guided biopsy from the area-of-interest by proceduralists in the Intervention Radiology department.

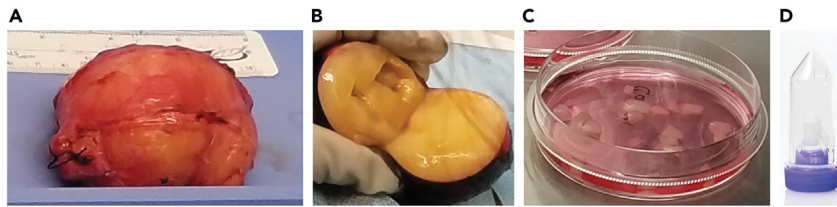


Figure 1. Illustration of tissue processing to achieve single-cell suspension from fresh nerve sheath tumor

(A) Example NF1-associated nerve sheath tumor obtained from the operation room.
(B) Picture of an inked and transected tumor with a $0.5 \times 0.5 \times 0.5 \text{ cm}^3$ cube of tissue acquired from the tumor.
(C) Picture of minced tumor with the presence of Tissue Dissociation Media in a petri dish.
(D) Picture of a c-tube that can be used on a gentleMACS™ dissociator. This tube can be directly placed in the incubator for shaking and enzymatic dissociation.

- ii. Once a core needle biopsy is obtained, immediately place the biospecimen in DMEM in a 15 mL conical tube.

Optional: Multi-regional sampling may be necessary if there is grossly visible heterogeneity (regional coloring or texture of the resected tumor) or magnetic resonance imaging (MRI) indicated that significant heterogeneity exists within the same tumor. Process the samples from different regions of the same tumor separately in the following steps.

△ CRITICAL: It is important to keep appropriate labeling and annotation for each specimen throughout the procedure. Pictures and annotations should be properly documented.

Generating single-cell suspensions of nerve sheath tumor

⌚ **Timing:** 2 h

This step describes how to use enzymatic dissociation to generate a single-cell suspension of nerve sheath tumors.

3. Dissection of the biospecimen and curation of resected tumor
 - a. Spray the outside of the conical tube with 70% ethanol and open it under a laminar flow hood. The tissue will be processed in the hood.
 - b. Transfer the biospecimen to a 60 mm cell culture plate. Using sterile surgical scalpel and tweezers, dissect the tumor into pieces of $2 \times 2 \times 2 \text{ mm}^3$ (Figure 2)
 - i. Fix one piece of the biospecimen in 10% buffered formalin solution at 25°C for a maximum of 24 h before transferring to 80% alcohol.

Note: Process this piece for the paraffin embedded tissue block. This piece can be used for downstream validation experiments.

- ii. Place two to three pieces individually in Eppendorf tubes and snap freeze in liquid nitrogen.

Note: Store these pieces at -80°C . They may be used for bulk sequencing or other experiments.

- iii. Mince the remaining tumor into $0.5 \times 0.5 \times 0.5 \text{ mm}^3$ pieces in the cell culture plate.

△ CRITICAL: When processing a core needle biopsy, no dissection or curation is needed. Directly transfer the biopsy biospecimen into a c-tube for enzymatic dissociation.

4. Enzymatic dissociation

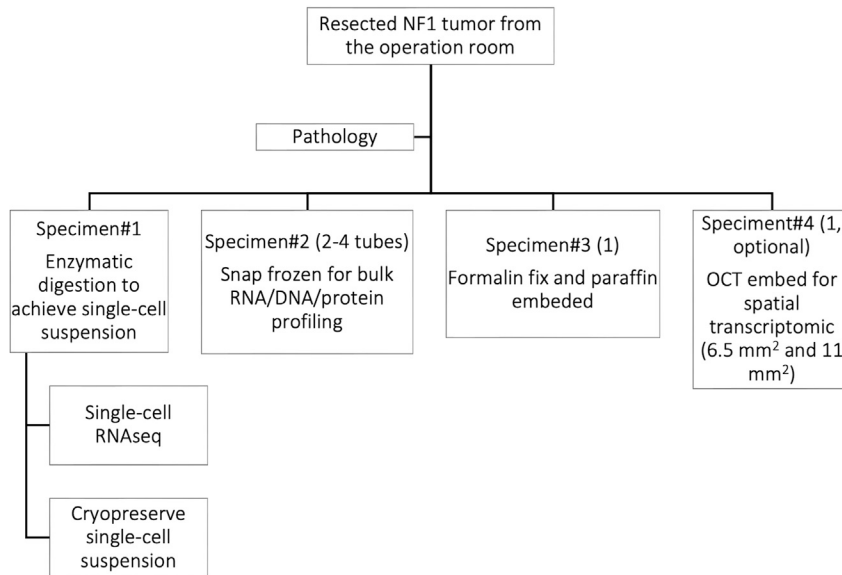


Figure 2. Schematic of the workflow of NF1-associated nerve sheath tumor processing from the surgical suite to the laboratory

- a. For each sample to be dissociated, prepare 10 mL of complete Tumor Dissociation Media.
- b. Use a 10 mL serological pipette to add the Tumor Dissociation Media to the minced tumor and transfer them to a c-tube (Miltenyi).
- c. Place the c-tube on the gentleMACS™ dissociator and run “hTumor_2.1” twice.
- d. Shake the c-tube at 37°C for up to 40 min at 200 rpm.

△ **CRITICAL:** To process a core need biopsy sample, reduce the incubation time for enzymatic dissociation in step 4d to 20 min.

- e. Place the c-tube on the gentleMACS™ dissociator and run “hTumor_3.1” twice.
- f. Place a 40 µm cell strainer on a 50 mL conical tube under the laminar flow hood.
- g. Use a 10 mL serological pipette to agitate the dissociated sample in the tube under the hood to agitate the tumor into single-cell suspension.
- h. Transfer the suspension in the c-tube (dissociated sample may contain small pieces of tumor chunk) onto the cell strainer.
- i. Use the plunger from a 10 mL syringe to push the tumor chunks on the cell strainer.
- j. Rinse the cell strainer with 10 mL DMEM twice. The filter-through will contain ~ 30 mL of dissociated tumor cells (Figure 3).
- k. Add 60 µL of sterile 0.5 M EDTA solution into the filter-through single-cell suspension and mix by inverting the tube.
- l. Centrifuge the cells at 300 g for 5 min to collect the cell pellet.

△ **CRITICAL:** Always add EDTA to the single-cell suspension to a final concentration of 1 mM before spinning the cells to prevent aggregation.

- m. Using a 10 mL serological pipette, carefully remove all but 0.5 mL of the media. Resuspend the cell pellet.
- n. Count the cells using Acridine Orange/Propidium Iodide (AO/PI) fluorescent dye by mixing 20 µL cell suspension with 20 µL dye (Nexcelom) and loading 20 µL of the mixture to the disposable slide (Nexcelom).

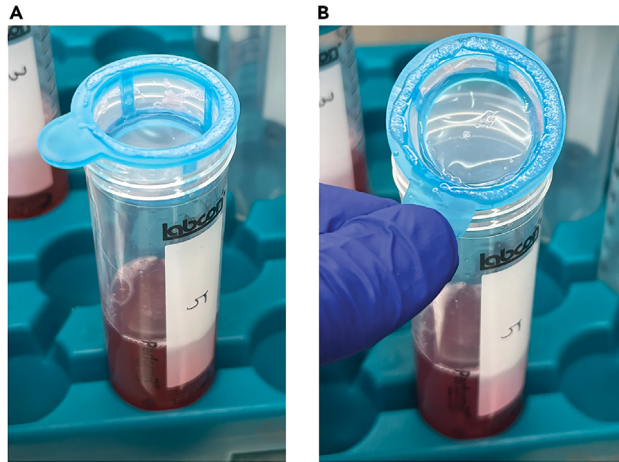


Figure 3. Illustration of using cell strainer to ensure the generation of single-cell suspension

(A) Picture of the setup of a 40 µm cell strainer on an opened 50 mL conical tube.

(B) After using a syringe plunger to mechanically force the remaining tissue chunks through the cell strainer, there should be minimal residual of tissue on the cell strainer.

- o. Insert the slide into the chamber of a fluorescent cell counter.

Note: Cellometer Auto 2000 was used in this study but other automatic cell counter or manual counting using a hemocytometer can also be used.

Note: Live cells fluoresce green fluorescent and dead cells fluoresce orange fluorescent.

Note: The automatic cell counter counts the number of green fluorescent cells as live cells and the number of orange fluorescent cells as dead cells.

- p. Calculate the cell concentration in the single-cell suspension using a dilution factor of 2.
- q. Wash the cells with 20 mL of DMEM and repeat steps l-p.
- r. Determine the cell concentration and viability again using AO/PI dye and a fluorescent cell counter (Figure 4).
- s. Adjust the cell concentration to be in the range of 700–1200 live cells per microliter in DMEM before proceeding to the single-cell sequencing.
- t. Process the cells immediately for single-cell capture.

△ CRITICAL: The success of the downstream single-cell RNA sequencing depends on good preparation of single-cell suspension with the viability above 70% and devoid of cell debris and dead cells.

Optional: If a gentleMACS™ dissociator is not available in the lab, use a 10 mL serological pipette to mix and agitate the minced tumor in Tumor Dissociation Media.

Optional: If significant amount of red blood cells exists in the sample, which will appear pink in the cell pellet, add 10 mL ACK lysing buffer to the resuspended cells after step 4m and incubate the sample at room temperature for 5 minutes, followed by PBS wash twice.

Optional: If the cell viability is less than 60%, an optional dead cell removal step maybe added after step 4p. Commercially available dead cell removal kit (Miltenyi Biotec) has been used and proved to be successful in enrichment of live cells. Be cautious that up to 50% of total cell loss should be expected using the dead cell removal kit.

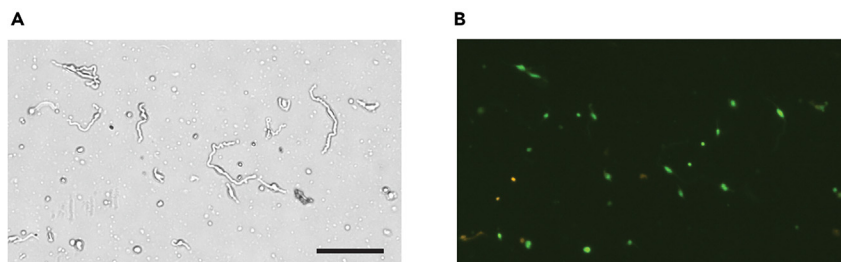


Figure 4. Example of cell count and viability determined by AO/PI staining on a fluorescent cell counter

(A) Bright field image. Note that the “string”-shaped cells are neoplastic Schwann cells isolated from a NF1-associated nerve sheath tumor.

(B) Fluorescent image showing live nucleated cells stained by Acridine Orange (green) and dead nucleated cells stained with Propidium Iodide (orange). Scale bar = 200 μ m.

Optional: It is possible to increase the content of Schwann cells in the dissociated single-cell suspension without affecting the downstream single-cell sequencing. We have previously identified that CD56 (encoded by *NCAM1*) is a unique cell surface marker expressed by mouse¹⁴ and human¹ Schwann cells. Using a CD56 antibody-based selection kit (STEMCELL), it is possible to enrich for Schwann cells isolated from the tumor for downstream single-cell sequencing.

Single-cell RNA sequencing using the 10x platform

⌚ Timing: 1–2 weeks

This step describes the procedure for 3' gene expression profiling of single cells including single-cell capture using 10x chromium platform, library preparation and sequencing. The recommended user guide “CG000315_ChromiumNextGEMSingleCell3-_GeneExpression_v3.1_DualIndex__RevE.pdf” is followed.

5. Single-cell capture.

- Count the cells again using fluorescent automated cell counter to determine viability and concentration before loading the cells on to a NextGEM chip for single-cell capture.
- If necessary, adjust the cell concentration to be in the range of 700–1200 live cells/ μ L by diluting or concentrating the cells from previous step.
- Count the cells one more time to check the cell concentration and viability just before loading.
- Load the cells with the target recovery of 6000 cells per capture lane.

Note: For most tumors processed in this project, two capture lanes per tumor sample are used, targeting 12,000 cells per tumor sample.

⚠ CRITICAL: Make sure not to introduce air bubbles while loading the cell suspension and other reagents into the wells.

⚠ CRITICAL: Before loading the cells for capture, make sure to check the viability and concentration of the samples one more time.

6. Library preparation.

- After the GEM generation, check the consistency and volume of the GEMs.

Note: If the quality of the GEMs is not as expected, for example (1) low volume of GEMs is observed, (2) GEMs appear less cloudy, or (3) transparent liquid is observed instead of the cloudy GEMs, it indicates either a wetting failure or partial emulsification has occurred.

△ **CRITICAL:** It is necessary to repeat the capture if the quality of the GEMs is not as expected. Before loading the cells for recapture, make sure to completely resuspend and count the cells again.

△ **CRITICAL:** After GEM formation, pay close attention to the volume, opacity, and consistency of the GEM for a clog or possible wetting failures.

- b. Proceed to GEM-RT incubations immediately after GEM generation.
Safe pause point: store the tubes at 4°C for up to 72 h or at –20°C for up to a week.
- c. Process the GEMs through GEM recovery, cleanup with Dynabeads, and prepare the cDNA.
Safe pause point: store the cDNA at 4°C for up to 72 h or at –20°C for up to 4 weeks.
- d. Check the quality and concentration of cDNA using one of the following quantification methods: Bioanalyzer, TapeStation, or Labchip.

Note: The quality and concentration of the cDNA provide some insights about the progress of the assay and helps for troubleshooting later if needed.

- e. Continue with final library preparation from cDNA that includes fragmentation, end repair and A-tailing, adaptor ligation, size selection using SPRIselect beads and sample index PCR.
Safe pause point: store the final library at 4°C for up to 72 h.
- f. After the completion of sample index PCR, perform one additional round of size selection using SPRIselect beads.
Safe pause point: store the tubes at 4°C for up to 72 h or at –20°C for long-term storage.
- g. Check the quality and concentration of the final library with a Bioanalyzer, TapeStation, or Labchip.

△ **CRITICAL:** Pay close attention to the size distribution and concentration of the final library and the size of the adaptor dimer peak.

7. Sequencing.

- a. The Final 3' gene expression dual indexed libraries are standard Illumina paired end constructs.

Note: Read 1 is 28 base pair (bp) long which encodes 16 bp 10x cell barcode and 12 bp unique molecule index (UMI). Read 2 is 90 bp long which contains the cDNA fragment that is later aligned to the reference genome to indicate the expressed RNA. The sample index sequences, i5 and i7, are 10 bp long each. They allow the pooling of several 10x cDNA libraries for sequencing.

- b. Calculate the molarity of the libraries using the concentration and the size of the final library.
- c. Prepare 2 nM library for each cDNA library, pool the 2 nM libraries from multiple 10x cDNA libraries and re-check the molarity.
- d. Dilute the pooled libraries to 1 nM as the final loading concentration.

Note: The final loading concentration is dependent on the type of Illumina sequencer used, and it needs to be optimized for each sequencer.

- e. Sequence the pooled libraries using an Illumina sequencer.

- f. When sequencing multiplexed 10x libraries, perform an initial shallow sequencing run to normalize the libraries so that each library will be sequenced at a similar molarity.

△ **CRITICAL:** The flow cell to use for running the libraries depend on the number of multiplexed libraries and the number of cells targeted. For example, for five multiplexed libraries and each with a target of approximately 6000 cells, the sequencing depth will be: 50,000 reads × 6000 cells × 5 libraries = 1.5 billion reads. NovaSeq S2 flow cell will yield approximately 1.3–1.6 billion single-end reads.

▮▮ **Pause point:** According to the manufacturer's protocol, safe pause points are available after the fresh single-cell suspension has been captured at steps 6b, 6c, 6e, and 6f.

△ **CRITICAL:** The time to complete this step varies based on the number of samples. The more samples captured and to be sequenced, the longer it will take to complete this step. One to two weeks are sufficient to process a typical 2 lanes of single-cell capture from one tumor sample.

Integrative data analysis, marker genes identification, and cell type annotation

⌚ **Timing:** 24 h

This step describes how to perform basic integrative analysis, marker gene identification and cell type annotations of single-cell RNAseq data generated from NF1 nerve sheath tumors.

8. Use the standard 10x Genomics cellranger (version 6.0.0) pipeline to extract fastq files and to perform data processing.
 - a. Demultiplex the raw base call files (BCL) generated by the Illumina sequencer into fastq files using cellranger mkfastq.
 - b. Run cellranger count pipeline for alignment to the reference genome (refdata-gex-GRCh38-2020-A), filtering, barcode and UMI counting.
9. Apply SoupX⁷ to the count matrix of each capture lane to correct the ambient RNA contamination in the data.
 - a. Load the count matrix generated from cellranger.
 - b. Manually specify the contamination fraction to 10%, 20%, 30%, and 40%.
 - c. Generate corrected count matrix to be used in downstream analysis.
 - d. Visually examine the expression of known markers of each cell type generated from using each contamination fraction. For example, B cell marker CD79B should be expressed in B cells but not the other cell types.
 - e. Once a desired contamination fraction is determined, use this contamination fraction to generate the corrected count matrix to be used in the next step.
10. Apply Scrublet⁸ to the SoupX-corrected read count matrix for the prediction of doublets in the data.
 - a. Based on the number of cells captured per capture lane, a predicted doublet rate is calculated as the following:

$$\text{Predicted doublet rate (\%)} = \frac{\text{number of cells captured}}{10000} \times 0.8$$

- b. Use the calculated predicted doublet rate on a per capture lane basis in function scr.Scrublet in Scrublet. This step will define the expected_doublet_rate.
 - c. Remove marked doublets before advancing to the next step in this data analysis pipeline.
11. Use Seurat¹⁵ for the integrative analysis of data from multiple captures to correct for potential batch effect.

- a. Remove cells with low quality ($nFeature_RNA < 500$, $nFeature_RNA > 6000$, or percent of mitochondrial gene fraction $> 20\%$).
 - b. Normalize corrected gene counts using the “LogNormalize” method with the scale.factor set to 10000.
 - c. Calculate the top 3000 most variable genes using the method “vst”.
 - d. Assign the cell cycle identity of each cell using the function CellCycleScoring.
 - e. Identify anchors using the FindIntegrationAnchors function with the setting “dims = 1:40”.
 - f. Apply anchor-based reciprocal principal component analysis (rPCA)¹⁶ to integrate data from different capture lanes.
 - g. Scale the data with the unwanted source of variations regressed out (“percent.mt”, “nCount_RNA”, “nFeature_RNA”, “S.Score”, “G2M.Score”).
 - h. Utilize the standard dimensional reduction for the clustering analysis using the RunPCA (npcs = 40), RunUMAP (reduction = “pca”, dims = 1:40), FindNeighbors (reduction = “pca”, dims = 1:40), and FindClusters (resolution = 0.8) functions in Seurat.
 - i. Identify cluster biomarkers by finding the differentially expressed genes in each cluster with comparison to all other clusters, using the FindAllMarkers function in Seurat with the setting “only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25” to derive the most highly expressed marker genes of each cluster.
12. Manually annotated cell types based on the identified marker genes in each cluster.

Optional: Several other R-based or Python-based tools are available for integrative analysis of single-cell RNA sequencing data. Users may choose any suitable bioinformatic tools for these steps.

Optional: Instead of manually specifying a contamination fraction, the function autoEstCont can be used in SoupX to automatically identify a desired contamination fraction (rho).

Building a normal Schwann cell developmental trajectory

⌚ Timing: 2 weeks

This step describes how to build a normal Schwann cell developmental transcriptional trajectory using publicly available single-cell RNAseq data. All source code used in this step can be found on: https://github.com/vishakagopalan/nf_hackathon.

13. Data download and processing.
 - a. Identify publicly available single-cell RNAseq data obtained from mouse normal developing Schwann cells. Download the read count matrices of the following data from GEO database.
 - i. Obtain data of neural crest stem cells at embryonic day 9.5 (E9.5) from GSE129114.
 - ii. Obtain data of Schwann cell precursors at E12.5 and E13.5 from GSE150150.
 - iii. Obtain data of mature myelinating and non-myelinating Schwann cells from adult mice from GSE142541.

Optional: Emerging single-cell RNAseq datasets of neural crest stem cells, Schwann cell precursors, as well as other developing and differentiated Schwann cell subtypes are available to be integrated. These datasets can also be used to build the normal Schwann cell developmental trajectory.

- b. Use published cell-type annotations for GSE129114 (Table S9 in source publication) and GSE142541.
- c. For GSE150150, perform batch correction and clustering using the default parameters in Seurat, and identify annotated cell types based on marker genes employed in the source publication.

- d. Merge all datasets together in Seurat.
- e. Use the library size (sequencing depth in each cell), cell cycle status, and batch as co-variables to estimate the p-value of differential expression for each gene.

14. Trajectory analysis in Monocle3.

- a. Down-sampling of reads from datasets that contain a much larger library sizes is required.

Note: Among the three datasets that we use in this study, GSE129114 and GSE150150 are downsampled to match the library size distribution of cells in GSE142541.

- b. Use Monocle3 to construct a trajectory from the merged data.
 - i. Merge the three datasets after the down-sampling.
 - ii. Perform batch correction using the internal function of Monocle3.
 - iii. Use E9.5 neural tube cells as the root state of the trajectory (these cells will be assigned a pseudotime value of 0) to compute pseudotime values of the remaining cells.

15. Regulon analysis in SCENIC.

- a. Regulons are defined as a target gene set for each transcription factor, active in each developing cell type, based on the presence of binding sites within 10 kilobases of the transcription starting site of expressed genes.
- b. Retain transcription factors whose mean regulon activity is higher than 0.03 in at least one of the cell types for future analysis.

16. Determine markers of developing Schwann cells using the FindAllMarkers function in Seurat.

17. Compute cell cycle status (G2M, S, or G1 phase scores) using AddModuleScore.

Note: The cell cycle status of each cell is considered as co-variables for determining the p-values of the log-fold changes of each differentially expressed gene.

Comparative analysis between neoplastic Schwann cell and malignant peripheral nerve sheath tumor cell

⌚ Timing: 6 h

This step describes how to transcriptionally project the neoplastic Schwann cells onto the normal Schwann cell developmental trajectory. All source code used in this step can be found on: https://github.com/vishakagopalan/nf_hackathon.

18. Compute at most 300 signature genes of each cell type.

Note: Signature genes of the developing Schwann cells are markers of each cell type from step 16 that have an adjusted p-value of less than 0.1.

19. Comparison between Schwann cells and MPNST malignant cells.

- a. Derive marker genes of neoplastic Schwann cells and MPNST malignant cells from the integrative analysis of patient nerve sheath tumors in step 12. Specifically, using the function FindMarkers with the setting (only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25), derive the most highly expressed marker genes in both malignant and Schwann cells.
- b. As part of the function of SCENIC package, score the activity of each of the signature gene sets (signature genes derived from step 15) in each cell using the AUCell function.
- c. Compare the AUCell activities of each developmental Schwann cell types between malignant MPNST cells and Schwann cells from the patient MPNST single-cell dataset using two-sided Wilcoxon test.

Table 1. Cell numbers of the NF1 tumor captured and presented in this study at each stage of the quality control

	Initial cell number	Cell number post Scrublet	Cell number post filtering
Primary MPNST	27,784	25,832	20,469
Metastatic MPNST	47,442	44,537	35,803

EXPECTED OUTCOMES

This protocol is intended for the identification of intra-tumoral heterogeneity of nerve sheath tumors using single-cell RNA sequencing on the 10x Genomics platform. Depending on the type of specimen, approximately 50,000 (one core needle biopsy) – 1,000,000 ($2 \times 2 \times 2 \text{ mm}^3$ tissue from a resected tumor) live cells are expected to be obtained from one patient nerve sheath tumor. Cell viability is determined with AO/PI staining on a fluorescent automatic cell counter, ranging from 70%–95% (Figure 4). Typically, two lanes of single-cell suspension are captured from each tumor, aiming to recover 12,000 live cells.

Using the Tissue Dissociation Buffer coupled with shaking at 37°C has been an efficient method to dissociate fresh nerve sheath tumors. With the help of an automatic dissociator (gentleMACS™ from Miltenyi Biotec) before and after the incubation, minced tumor pieces are adequately mixed with the Tissue Dissociation Buffer. If an automatic dissociator is not available, agitation with a 10 mL serological pipette can be used to mix the sample. Minimal residual tissue chunks should be observed after the dissociation when the sample is filtered through a 40 μm cell strainer (Figure 2B). Mechanical force of the residual tissue through the cell strainer using a syringe plunger followed by rinsing the strainer with DMEM is helpful to increase the yield without any compromise of cell viability.

Schwann cells are considered the cell-of-origin of NF1-associated nerve sheath tumors. The content of Schwann cells ranges between 1%–80% in the dissociated single-cell suspension. We have previously identified that CD56 (encoded by *NCAM1*) is a unique cell surface marker of mouse¹⁴ and human¹ Schwann cells. Using a CD56 antibody-based selection kit, we have previously enriched for CD56-positive human Schwann cells in an atypical neurofibroma, followed by successful single-cell sequencing on a 10x platform. This method increased the Schwann cell content from 30% to over 90% in the tumor and was proved not to affect the downstream single-cell sequencing.

Single-cell sequencing on the 10x Genomics platform has been adapted in this protocol. Using the commercial kit and following the manufacturer's protocol will generate reliable and reproducible single-cell sequencing data.

This protocol provides a general guideline for single-cell RNAseq data analysis as presented in Zhang et al.¹ To correct potential batch effects that is caused by captures, samples, cell cycle status of the cell, as well as other variables, anchor-based integration¹⁶ should be performed when merging sequencing data from multiple biospecimens, followed by dimension reduction and cell clustering. The cell numbers of the NF1 tumors included in the study¹ at each of the quality control steps are listed in Table 1. The integrated data is processed in the downstream analysis so that the shared cell populations (cell types) can be directly compared between pathological modalities. Patient-specific or capture-specific cell populations should not dominate the discovery of intratumoral heterogeneity within these tumor datasets.

LIMITATIONS

This protocol is intended to process fresh nerve sheath tumors that are directly obtained from the surgical suite. Timely acquisition and processing of the biospecimen are required to preserve the transcriptome and maintain viability of the cells. Typically, a tumor is processed within 15 min after it is acquired, and the enzymatic dissociation should not last more than 2 h. We do realize that a timely procedure may not be available given the unforeseen outcome of a complicated surgery. While not optimal, to best preserve

the freshness of biospecimens when dissociation is not immediately feasible, placing the freshly obtained sample in the MACS™ tissue storage solution (Miltenyi Biotec) at 4°C can maintain the cell viability for up to 48 h. If needed, single-nuclei RNA sequencing can also be employed in frozen tumor samples. However, the data integration and compatibility will need to be further explored when handling both single-cell and single-nuclei RNA sequencing data are used.

Based on previous experience and given the tissue composition of nerve sheath tumors, the recipe of enzymatic cocktail is composed of dispase II, collagenase I, and DNase I. However, this recipe maybe highly tissue-specific and may not work efficiently for other tissue types to generate single-cell suspension. To use this protocol in other tissue types, optimization of the enzyme cocktail and time for incubation time is necessary to ensure the yield and cell viability.

In order to build a normal Schwann cell developmental trajectory, we used publicly available single-cell sequencing data from mouse embryos (for neural crest stem cell subtypes and Schwann cell precursors), newborns (for immature Schwann cells), and adult mice (for differentiated Schwann cells). Mouse gene symbols were converted to the available human equivalent before this integrated dataset was used inform the difference between neoplastic Schwann cells and malignant cells. However, there are known discrepancies of gene function in mouse versus human during the development. Future work integrating developing human Schwann cells from the different developmental stages is warranted to better understand the de-differentiated phenotype that was observed in human MPNST cells.

Finally, a useful addition to these analyses would be the inclusion of Schwann cells from normal nerve. However, human normal nerve from the same patient (sites where this is no neurofibroma) or healthy donors (normal *NF1* status genetically) are typically spared in clinical debulking surgeries. On the other hand, single-cell RNAseq of mouse *NF1*-associated nerve tumors often contain cell clusters of neurons.¹⁴ The different approaches used in human and mouse sampling should be taken into considerations when discrepancies are observed.

TROUBLESHOOTING

Problem 1

The viability of cells determined by the fluorescence cell counter is lower than 70% (step 4).

Potential solution

Cell viability will decrease as time passes after the surgical resection. To reduce cell loss and death, the procedures of sample acquisition, dissection, and enzymatic dissociation need to be completed within 1–2 h after the tumor comes out of the operation room. If live cells are surrounded by cell debris, additional cell washes with DMEM may help clean up the cell debris. If a large quantity of dead cells (orange) determined by the fluorescence cell count exist, a step of dead cell removal using a magnetic separator (Miltenyi Biotec) may be employed. Note that any additional dead cell removal steps will cause significant cell loss (~50%). It is not recommended to perform dead cell removal if limited number of cells are available.

Problem 2

Large quantity of red blood cells exists in the sample with the cell pellet appearing pink (step 4).

Potential solution

It is possible that red blood cells exist in large quantity in a single-cell suspension, especially when the sample is obtained as a core needle biopsy. As determined by a fluorescent cell counter, red blood cells appear in the bright field image as small round cell-like particles, but they are not stained with AO/PI dye since they do not have nuclei. Presence of these cells will significantly hamper the result and it is critical to sufficiently remove them if the cell pellet appear pink or red after step 4l. It is recommended to use 10 mL ACK lysis buffer to resuspend the bloody cell pellet, followed by

5 min incubation at room temperature and two times of washes. Cell concentration and viability need to be determined again before proceeding to the subsequent steps.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jack F. Shern (john.shern@nih.gov).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate a new dataset. The single-cell RNA sequencing dataset of the human neurofibromatosis type 1 (NF1) malignant nerve sheath tumors mentioned in this protocol was previously described by Zhang et al. (2022).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

X.Z., V.G., J.F.S., and N.S. wrote the manuscript. X.Z. developed and implemented the pipeline of processing clinical samples for single-cell sequencing described here and performed the experiments and analysis of the single-cell sequencing data of patient NF1 tumors. V.G. performed analysis of the public single-cell sequencing data from mice. N.S. provided technical support for single-cell sequencing of clinical samples. J.F.S. supervised the single-cell sequencing study at the POB. S.H. and J.F.S. supervised the study. All authors commented on and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

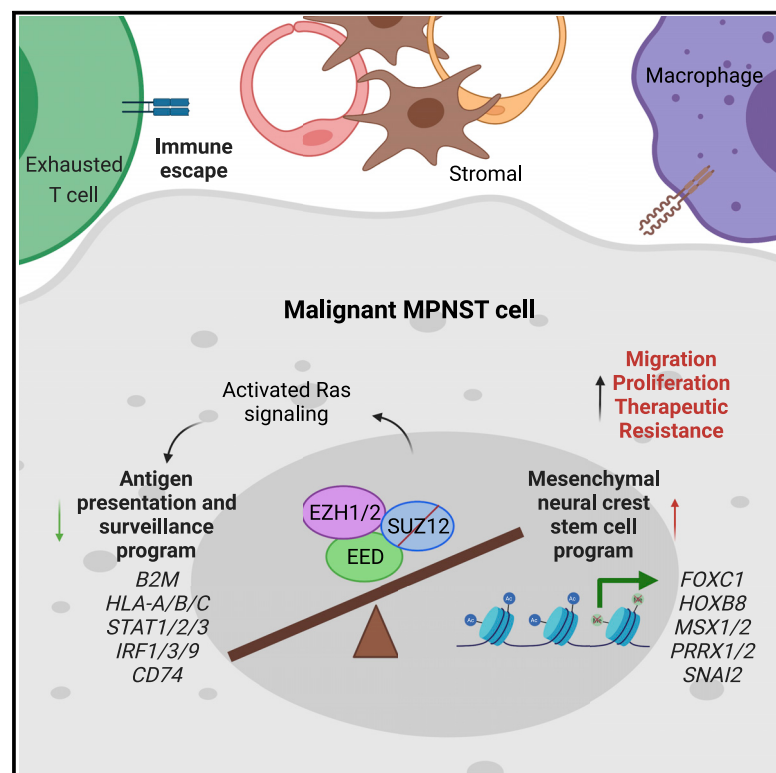
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Single-cell sequencing reveals activation of core transcription factors in PRC2-deficient malignant peripheral nerve sheath tumor

Graphical abstract



Authors

Xiyuan Zhang, Hannah E. Lou, Vishaka Gopalan, ..., Michael C. Kelly, Sridhar Hannenhalli, Jack F. Shern

Correspondence

john.shern@nih.gov

In brief

Zhang et al. provide evidence that PRC2 loss activates cell-fate-determining transcription factors by recruiting active enhancers and dampens type I interferon signaling and antigen presentation through transcriptional cross talk with the hyperactivated Ras. These observations are supported by integrative analysis of single-cell sequencing of patient MPNST samples.

Highlights

- PRC2 loss activates bivalent genes via transcriptional recruitment of active enhancers
- PRC2 loss activates core TFs required to sustain the MPNST oncogenic program
- PRC2-null MPNSTs mimic Schwann cell progenitors and reduce antigen presentation
- scRNA-seq reveals heterogeneity within the MPNST microenvironment



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Article

Single-cell sequencing reveals activation of core transcription factors in PRC2-deficient malignant peripheral nerve sheath tumor

Xiyuan Zhang,¹ Hannah E. Lou,¹ Vishaka Gopalan,² Zhihui Liu,¹ Hilda M. Jafarah,¹ Haiyan Lei,¹ Paige Jones,¹ Carly M. Sayers,¹ Marielle E. Yohe,¹ Prashant Chittiboina,³ Brigitte C. Widemann,¹ Carol J. Thiele,¹ Michael C. Kelly,⁴ Sridhar Hannenhalli,² and Jack F. Shern^{1,5,*}

¹Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

²Cancer Data Science Laboratory, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

³Neurosurgery Unit for Pituitary and Inheritable Diseases, National Institute of Neurological Diseases and Stroke, Bethesda, MD 20892, USA

⁴Center for Cancer Research Single Cell Analysis Facility, Cancer Research Technology Program, Frederick National Laboratory, Bethesda, MD 20892, USA

⁵Lead contact

*Correspondence: john.shern@nih.gov

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SUMMARY

Loss-of-function mutations in the polycomb repressive complex 2 (PRC2) occur frequently in malignant peripheral nerve sheath tumor, an aggressive sarcoma that arises from *NF1*-deficient Schwann cells. To define the oncogenic mechanisms underlying PRC2 loss, we use engineered cells that dynamically reassemble a competent PRC2 coupled with single-cell sequencing from clinical samples. We discover a two-pronged oncogenic process: first, PRC2 loss leads to remodeling of the bivalent chromatin and enhancer landscape, causing the upregulation of developmentally regulated transcription factors that enforce a transcriptional circuit serving as the cell's core vulnerability. Second, PRC2 loss reduces type I interferon signaling and antigen presentation as downstream consequences of hyperactivated Ras and its cross talk with STAT/IRF transcription factors. Mapping of the transcriptional program of these PRC2-deficient tumor cells onto a constructed developmental trajectory of normal Schwann cells reveals that changes induced by PRC2 loss enforce a cellular profile characteristic of a primitive mesenchymal neural crest stem cell.

INTRODUCTION

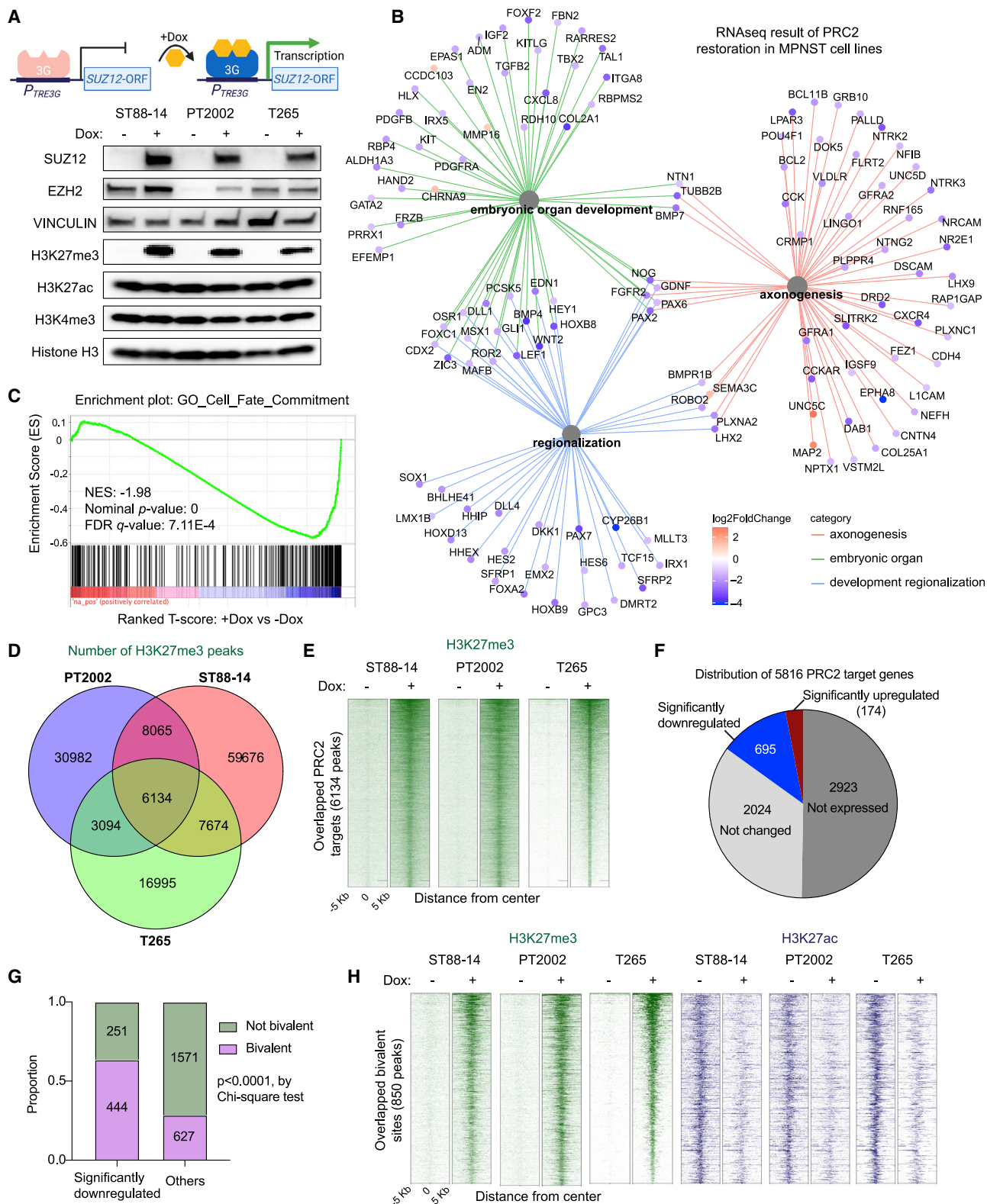
Polycomb repressive complex 2 (PRC2) is a multisubunit histone-modifying (epigenetic) protein complex that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27me3) and is required for proper maintenance of transcriptional repression of key genes during development and cell lineage determination (Bracken et al., 2006; Pasini et al., 2004; Xie et al., 2014). Not surprisingly, given its central role in gene regulation, cancer cells frequently corrupt PRC2 through a variety of mechanisms (Comet et al., 2016; Laugesen et al., 2016). Gain- or loss-of-function alterations of PRC2 in the cancer cell lead to a diversity of downstream consequences such as cellular proliferation (Piunti et al., 2014), invasion (Ren et al., 2012), resistance to apoptosis (Ariès et al., 2018), metastasis (Gupta et al., 2010), and epithelial-to-mesenchymal transition (Serresi et al., 2016). Interestingly, these tumor cell phenotypes frequently mirror the cellular characteristics observed during normal cellular developmental processes, which raises the intriguing prospect that mutation of PRC2 might enforce the corruption of a primitive cellular program.

Malignant peripheral nerve sheath tumors (MPNSTs) are soft-tissue sarcomas that arise predominantly in patients with neuro-

fibromatosis type 1 (NF1) and follow an aggressive clinical course. Currently, there is no effective medical therapy, and complete surgical removal is required to cure MPNST. Loss of a functional PRC2, through the mutation or deletion of one of its core components *SUZ12* or *EED*, is highly recurrent and present in 60%–90% of all MPNSTs (De Raedt et al., 2014; Lee et al., 2014; Zhang et al., 2014). In MPNST, the loss of PRC2 has a diverse set of consequences, including hyperactivated Ras signaling (De Raedt et al., 2014), increased expression of WNT pathway genes (Lee et al., 2014), and downregulation of genes involved in immune surveillance (Wojcik et al., 2019). In addition, gain of acetylated H3K27 (H3K27ac) accompanying the loss of H3K27me3 has been observed in PRC2-deficient MPNST (De Raedt et al., 2014; Lee et al., 2014; Wojcik et al., 2019). A mechanistic understanding of how the loss of PRC2 results in these diverse, but specific, consequences remains unresolved.

In this study, we sought to improve the basic understanding of PRC2 loss as an oncogenic mechanism in MPNST, and ultimately to define potential therapeutic targets. To accomplish this, we performed a detailed epigenetic and transcriptomic characterization of inducible PRC2 reconstitution cellular systems to discover genomic regions of bivalent chromatin that





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are dysregulated upon the loss of PRC2. Data from the engineered model systems were integrated with single-cell RNA sequencing (scRNA-seq) and single-cell whole-genome sequencing (scWGS) of PRC2-deficient primary and metastatic human tumors. Through the integration of these datasets, we uncovered that PRC2 loss enforces an immune-suppressive and lineage-specific oncogenic transcriptional program that is characteristic of a neural-crest-derived mesenchymal stem cell.

RESULTS

PRC2 restoration drives a genome-wide gain of H3K27me3 occupancy and transcriptional reduction at bivalent domains

To investigate the transcriptomic and epigenetic consequences of PRC2 loss in MPNST, we characterized a panel of human cell lines for their functional PRC2 status based on the presence or absence of H3K27me3 (Figure S1A). Three SUZ12-deficient cell lines were engineered to express a full-length SUZ12 open reading frame (ORF) or an empty vector (EV) under a doxycycline (Dox)-inducible promoter (Figure 1A). These models restored SUZ12 expression in a Dox-dose- and time-dependent fashion (Figures S1A–S1C). Physical reassembly of SUZ12 with EZH2 was validated by immunoprecipitation (Figure S1D), and functional reassembly of the PRC2 complex was confirmed by the induction of H3K27me3 in histone extracts (Figures 1A and S1C).

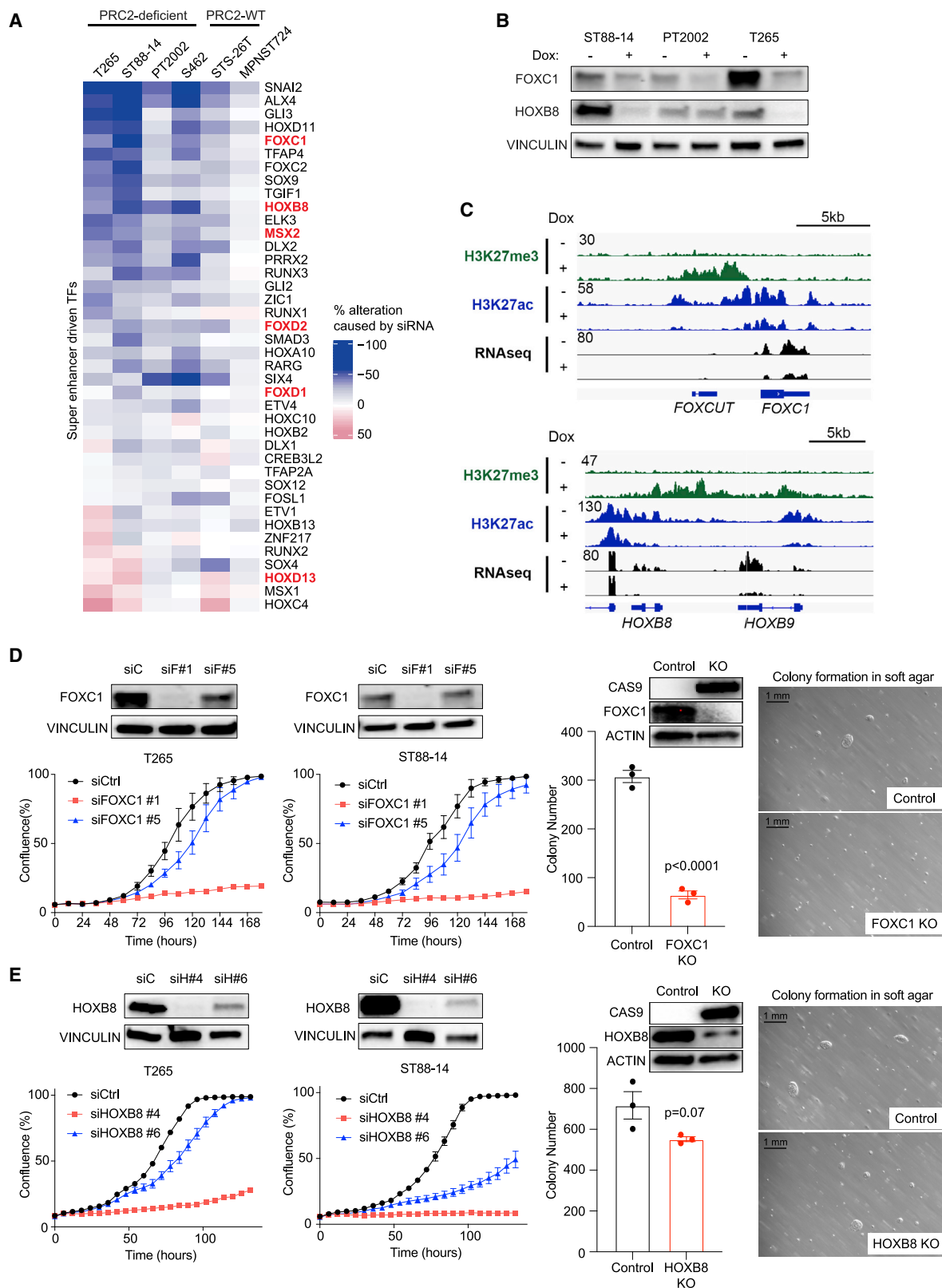
In PRC2-null T265 cells, PRC2 reconstruction by Dox-induced SUZ12 reexpression inhibited the cell growth in a dose-dependent manner (Figure S1E). Similar dose-dependent growth-inhibition effects were observed in the other PRC2-null MPNST cell lines as well (Figures S1F and S1G). To profile the transcriptomic consequences of PRC2 loss, we performed RNA-seq in this inducible system. A total of 1,683 and 1,357 genes were significantly downregulated and upregulated, respectively, upon the restoration of a functional PRC2 ($p_{\text{adj}} < 0.05$, Table S1). The dominant transcriptional alteration was the downregulation of genes involved in axonogenesis ($p_{\text{adj}} = 8.92\text{E}-19$), embryonic organs ($p_{\text{adj}} = 6.51\text{E}-10$), and development regionalization ($p_{\text{adj}} = 6.01\text{E}-12$) (Figure 1B; Table S1). Strikingly, multiple forkhead (FOX) or homeobox (HOX) transcription factors (TFs) that are critical for cell-fate specification were transcriptionally downregulated by PRC2, including *FOXC1* (log2 fold change [FC] =

-1.30 , $p_{\text{adj}} = 2.81\text{E}-05$), *FOXA2* (log2FC = -2.44 , $p_{\text{adj}} = 1.82\text{E}-06$), *HOXB8* (log2FC = -3.11 , $p_{\text{adj}} = 6.08\text{E}-11$), and *PRRX2* (log2FC = -1.29 , $p_{\text{adj}} = 1.33\text{E}-07$). Gene set enrichment analysis (GSEA) demonstrated that genes involved in “Cell_Fate_Commitment” were significantly repressed by PRC2 (enrichment score = -1.98 , $p = 0$; Figure 1C). These results indicated that the loss of PRC2 may contribute to MPNST tumorigenesis via activation of cell-fate-determining TFs.

To characterize the effects of PRC2 restoration on global gain in H3K27me3, 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 81,549, 48,275, and 33,897 significant H3K27me3 peaks in ST88-14, PT2002, and T265 cells, respectively (Figure 1D). Notably, there were 6,134 peaks common to all three cell lines, and 37,235 peaks were commonly detected in at least two of three cell lines (Figures 1D and 1E). A total of 5,816 genes were associated with these peaks, and interrogation of the RNA-seq for these genes demonstrated that most of these genes were either not expressed (2,923 genes, 50.3%) or transcriptionally unaltered (2,024 genes, 34.8%), regardless of the PRC2 and H3K27me3 status in the cells (Figure 1F). Of the 869 (14.9%) significantly altered genes, 695 (11.9%) were downregulated; and 174 (3%) were upregulated when a functional PRC2 was restored (Figure 1F). ChIP-seq of additional histone marks, including the mutually exclusive mark H3K27ac and bivalent chromatin mark H3K4me3, was also performed. Globally, H3K27ac was reduced in all three cell lines (Figure S1H). The decrease in H3K27ac and unchanged H3K4me3 were notable at sites of H3K27me3 (Figure S1I). Analysis of these sites demonstrated that genes that were both direct PRC2 targets and repressed with the gain of H3K27me3 were significantly enriched at bivalent sites ($p < 0.0001$; Figure 1G). Bivalent domains marked by both H3K27me3 and H3K4me3 are poised for rapid transcriptional control during development (Bernstein et al., 2006). A total of 850 common bivalent genomic sites were observed in the three cell lines (Figure 1H). Interestingly, the H3K27ac enhancer signal was drastically reduced as the genome regained H3K27me3, and this effect was accompanied by a modest decrease in H3K4me3 signal and chromatin accessibility measured by assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) (Figures 1H, S1J, and S1K).

Figure 1. PRC2 restoration drives genome-wide gain of H3K27me3 occupancy

- (A) Doxycycline (Dox)-induced reexpression of SUZ12 in MPNST cells restored trimethylated histone H3 lysine 27 (H3K27me3), as shown by western blotting.
- (B) Network plot demonstrating the connection of three significantly enriched pathways of the differentially expressed genes (DEGs) caused by PRC2 restoration. DEGs were derived from a combined analysis of the RNA-seq results obtained from all three PRC2-null MPNST cell lines, comparing samples with induced expression of SUZ12 to controls; $p_{\text{adj}} < 0.05$, $|\log_2 \text{fold change}| > 1$.
- (C) Gene set enrichment plot shows that PRC2 restoration significantly decreased genes in the “cell-fate commitment” pathway. NES, normalized enrichment score.
- (D) Venn diagram showing the number of overlapped H3K27me3 peaks in three MPNST cell lines upon PRC2 restoration.
- (E) PRC2 restoration drove the genome-wide gain of H3K27me3 occupancy as demonstrated by overlapped ChIP peaks in MPNST cells.
- (F) Pie chart showing the distribution of transcriptional alterations among the 5,816 genes associated with H3K27me3 peaks found to be common in at least two cell lines upon Dox induction. Dark gray, genes not expressed; light gray, genes expressed but not changed; blue, significantly downregulated; red, significantly upregulated; $p_{\text{adj}} < 0.05$.
- (G) Bar plot shows the enrichment of bivalent genes among the 695 downregulated genes upon PRC2 restoration.
- (H) Heatmap of ChIP-seq signals shows that PRC2 restoration caused gain of H3K27me3 and loss of H3K27ac at 850 common bivalent sites in MPNST cells.



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PRC2-regulated super-enhancer-driven TFs are critical for the survival of MPNST cells

Super enhancers (SEs) are characterized by long stretches of H3K27ac peaks co-occupied by BRD4 and MED; they drive active transcription and are often related to genes specifying cell identity (Hnisz et al., 2013; Whyte et al., 2013). Consistent with the reduced H3K27ac signals at bivalent domains, the total number of SEs decreased in two of three of our inducible systems as a result of PRC2 restoration (Figure S2A). Since SEs regulate cell-fate-determining TFs that can form a core transcriptional regulatory circuitry and are critical for cancer cell survival (Decaestecker et al., 2018; Durbin et al., 2018; Ran et al., 2018), we next sought to test if PRC2-regulated SE-driven TFs were essential for the survival of MPNST cells. To accomplish this, we identified TFs that were both highly expressed in human MPNST samples and associated with an autoregulated SE (as defined if its own binding motif was identified within its assigned SE) in the three inducible model systems (Figure S2B; Table S2). In total, we compiled a list of 40 MPNST-specific SE-driven TFs that were present in any of the three cell lines (Figure S2C; Table S2) and performed a small interfering RNA (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 17/40 (42.5%) of the TFs were critical for T265 cells (medium growth reduction rate >30%) and 22/40 (55%) of the TFs were essential for ST88-14 cells (Figure 2A; Table S2). Conversely, the knockdown of only one gene (*HOXC4*) in T265 and no genes in ST88-14 increased cell growth by greater than 30% (Table S2). Common essential TFs included *SNAI2*, which demonstrated a 50% growth reduction in five of six 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 (Figures 2B and 2C). 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* (Figure 2D) and *HOXB8* (Figure 2E), and this result was true when PRC2 was restored in these cell lines as well as in PRC2 wild-type (WT) cell lines (Figures S2D, S2E, and S2F). 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 (Figures 2D and 2E). Overall, these results suggest that PRC2-regulated TFs that are derepressed and SE driven as a result of PRC2 loss play a critical role in the survival of MPNST cells.

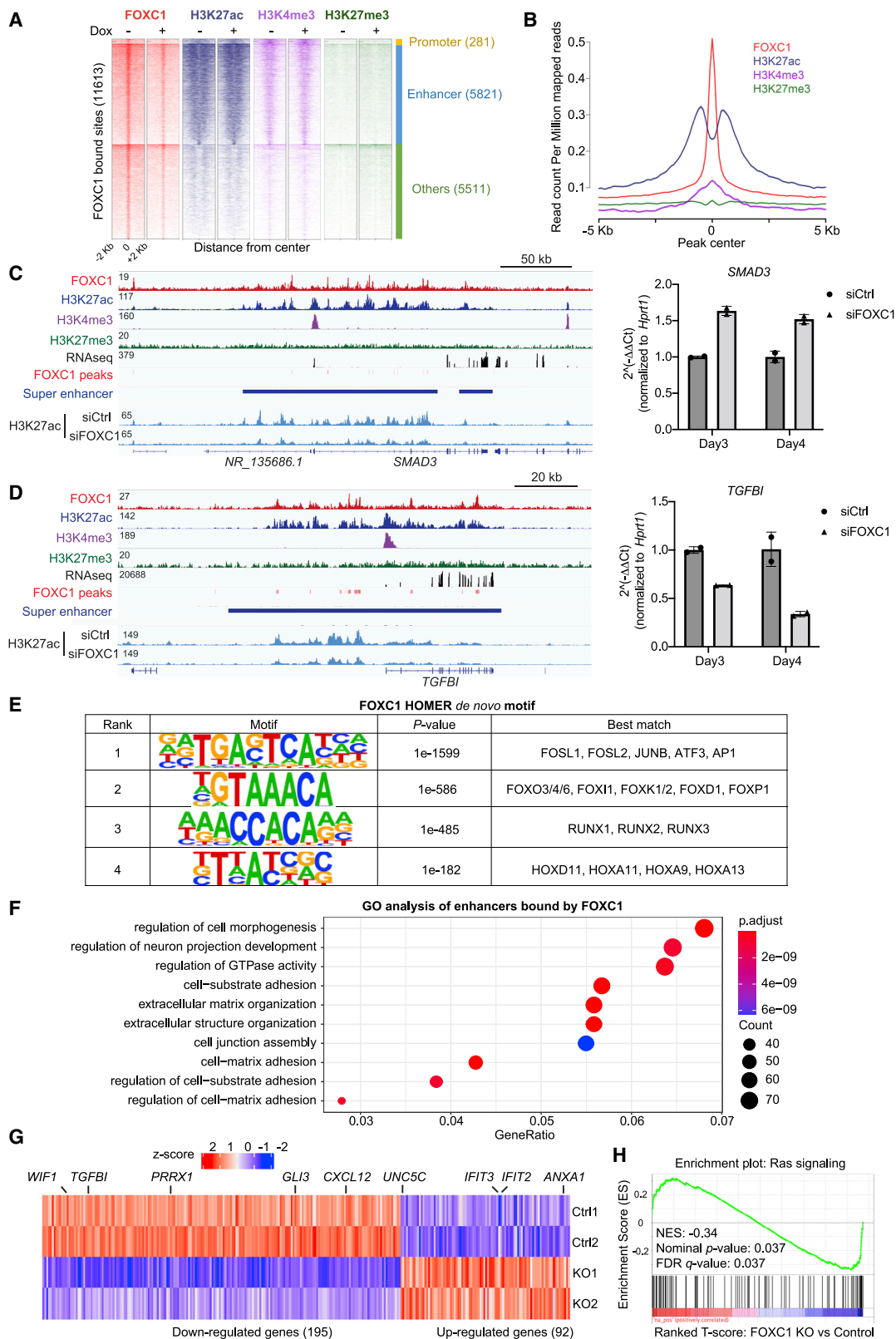
FOXC1 binds SEs in PRC2-null MPNST cells, enforcing oncogenic signaling

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 (Shang et al., 2018; Zhu et al., 2017), but its binding has not previously been assessed genome wide. *FOXC1*-specific ChIP-seq was performed to determine its genome binding in T265, ST88-14, and PT2002 cells with or without the Dox-induced PRC2 restoration. A total of 11,613 peaks ($p = 1.0E-05$), associated with 4,869 genes, were identified in PRC2-deficient T265 cells (Figures 3A and S3A). PRC2 restoration repressed *FOXC1* transcription (Figures 1B and 2C) and reduced its protein level (Figure 2B), as well as its genome-wide binding signal intensity (Figures 3A and S3C). However, residual *FOXC1* in PRC2-restored cells bound the same genomic loci, albeit with a reduced signal (Figures 3A, S3B, and S3C), and this was highly correlated in all three cell lines (Figure S3D). Among these peaks, only 2.8% (281 peaks) were present at gene promoters (Figures 3A and S3A), and 50.1% (5,821 peaks) of the discovered peaks overlapped with H3K27ac-based active enhancers as defined by Rank Ordering of Super-Enhancers (ROSE) (Lovén et al., 2013; Whyte et al., 2013) (Figures 3A and 3B). Notably, *FOXC1* binding at highly expressed genes overlapped with SEs decorated by long stretches of H3K27ac peaks near the gene body (Figures 3C and 3D). PRC2 restoration was noted to reduce *FOXC1* binding at these highly expressed genes (Figures S3E and S3F).

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) HOX domain-containing TFs (Figure 3E). Notably, like *FOXC1*,

Figure 2. PRC2-regulated super-enhancer-driven transcription factors are essential to the survival of MPNST cells

- (A) Heatmap showing the median percentage of reduction in proliferation when a candidate core TF was knocked down using three distinct siRNAs, in comparison with the control siRNA. Data are shown in the order of average median reduction from the highest to the lowest in four PRC2-deficient and two PRC2-WT cell lines. Data summarize two replicates per siRNA per cell line. Direct PRC2 targets are highlighted in red.
- (B) Western blot shows the reduction of protein levels of *FOXC1* and *HOXB8* caused by Dox-induced SUZ12 reexpression.
- (C) Example integrative genomics viewer (IGV) tracks around the *FOXC1* (top) and *HOXB8* (bottom) loci demonstrate the gain of H3K27me3, loss of super enhancer (SE), and transcriptional reduction accompanying the Dox-induced PRC2 restoration in T265 cells.
- (D) Cell growth assay using individual siRNAs targeting *FOXC1* in T265 (first panel) and ST88-14 (second panel). siC, siControl; siF#1, siFOXC1 #1; and siF#5, siFOXC1 #5. Quantification (third panel) and representative picture (fourth panel) of colony formation when *FOXC1* was knocked out in T265 cells by CRISPR-Cas9 are shown.
- (E) Cell growth assay using individual siRNAs targeting *HOXB8* in T265 (first panel) and ST88-14 (second panel). siH#4, siHOXB8 #4, and siH#6, siHOXB8 #6. Quantification (third panel) and representative picture (fourth panel) of colony formation when *HOXB8* was knocked out in T265 cells by CRISPR-Cas9 are shown. The average of five replicates is presented; mean $\pm$ SEM; scale bar, 1 mm.



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these TFs were highly expressed in MPNSTs and had a SE associated with the TF gene, and this SE was frequently bound by clusters of FOXC1 peaks, as shown for the *SMAD3* locus (Figure 3C). To further investigate the FOXC1-mediated regulation of these sites, we performed siRNA-mediated FOXC1 knockdown (Figure S3G) and evaluated gene expression and genome levels of H3K27ac by CUT&RUN assay. Knockdown of FOXC1 reduced H3K27ac both globally and at FOXC1 genomic binding sites (Figure S3H). Highlighted genes included *SMAD3*, *TGFBI*, and *RUNX2*, which showed reduction of H3K27ac signals at their identified SE (Figures 3C, 3D, and S3I). Interestingly, FOXC1 knockdown increased *SMAD3* (Figure 3C) and *RUNX2* (Figure S3I) expression while decreasing *TGFBI* (Figure 3D), indicating the potential for complex control of FOXC1 target genes. Interrogation of the motifs of these genes' H3K27ac peaks predicted that they bound one another's SE (Figures S3J and S3K). Therefore, we hypothesized that FOXC1, induced by PRC2 loss, plays a critical role in MPNST oncogenesis by participating in a core TF regulatory circuit (Assi et al., 2019; Somerville et al., 2015). Gene ontology (GO) pathway enrichment analysis of the FOXC1-bound enhancer-associated genes revealed neuron development and differentiation, extracellular matrix formation, and Ras signaling (Figure 3F; Table S3). RNA-seq of CRISPR-Cas9 knockout of *FOXC1* identified that 45% (195/436) of the downregulated and 24% (92/377) of the upregulated genes had corresponding FOXC1 ChIP-seq peaks (Figure 3G). Downregulation of the Ras signaling gene set, which has previously been described as a feature of PRC2 restoration (De Raedt et al., 2014), was also significantly downregulated with *FOXC1* knockout (enrichment score -0.34 , $p = 0.037$; Figure 3H). In summary, these findings indicated that FOXC1 participates in a core transcriptional regulatory circuit at SEs of highly expressed genes and drives MPNST oncogenesis by regulating critical pathways, including Ras (Figure S3L).

PRC2 restoration indirectly upregulates interferon signaling and antigen presentation

Consistent with its known repressive role, PRC2 restoration resulted in the dominant downregulation of aberrantly activated genes (Figure 1). However, 1,357 genes were significantly upregulated ($p_{\text{adj}} < 0.05$) when PRC2 was restored. Pathway enrichment analysis demonstrated that upregulated genes were

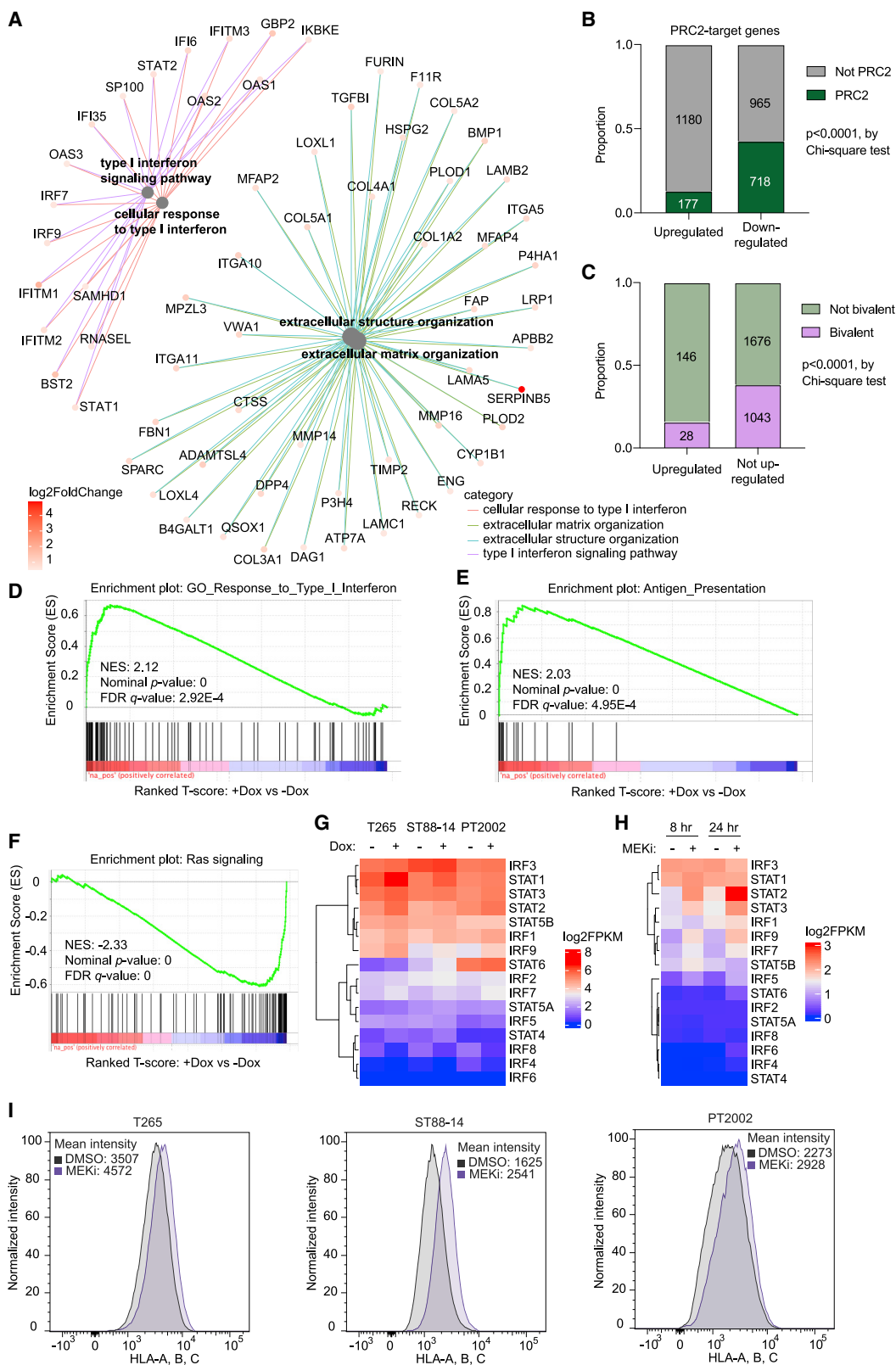
involved in type I interferon signaling ($p_{\text{adj}} = 1.0\text{E}-06$) and extracellular matrix organization ($p_{\text{adj}} = 5.6\text{E}-10$) (Figure 4A; Table S1). Consistent with the repressive function of PRC2, only 177 (13%) of the upregulated genes exhibited a corresponding gain of H3K27me3 signal, while 718 (43%) of the downregulated genes gained a H3K27me3 mark in the Dox-treated cells ($p < 0.0001$, Figure 4B). The 174 PRC2-targeted upregulated genes were not found to be enriched at bivalent regions compared with the other PRC2-targeted genes (16.1% versus 38.4%, $p < 0.0001$; Figure 4C). Taken together, these results indicated that the upregulation of genes involved in the type I interferon signaling and extracellular matrix organization occurred as an indirect effect of PRC2 restoration.

The downregulation of proteins involved in type I interferon signaling and antigen presentation has been reported in PRC2-null MPNSTs at the protein level compared with MPNSTs that retain PRC2 (Wojcik et al., 2019). Notably, the upregulated genes in our inducible model system mirrored this observation at the RNA level for these two processes (Figures 4D and 4E). We further confirmed the transcriptional observations at the protein level and found the induction of HLA-A, -B, and -C on the cell surface of PRC2-restored MPNST cells (Figures S4A and S4B), as well as an increase in multiple type I interferon signaling proteins (Figure S4C). To understand the apparent indirect mechanism underlying the induction of these genes, we analyzed 4,824 H3K27ac peaks associated with the upregulated genes in T265 cells (Figure S4D). The signal intensity of these H3K27ac peaks was significantly higher in the Dox-treated sample (Figure S4E), indicating the possible transcriptional regulation of these genes through recruitment of the transcriptional machinery to these regions. Motif profiling of these H3K27ac peaks demonstrated the presence of AP1 family TFs and, interestingly, an enrichment for the IRF family TFs as potential regulators (Figure S4F).

Hyperactive Ras has previously been shown to downregulate immune signaling (Gimple and Wang, 2019), so we hypothesized that this same mechanism might explain our observations. Indeed, in the inducible system, we observed the downregulation of the Ras signaling gene set as a direct consequence of PRC2 restoration (Figure 4F). Interestingly, upon PRC2 restoration, we observed the upregulation of several STAT and IRF family TFs (Figures 4G and S4C), which are responsible for the

Figure 3. PRC2-regulated super-enhancer-driven FOXC1 determines the cell lineage specificity in MPNST cells

- (A) 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 were calculated over 11,613 FOXC1 peaks that were segmented into three categories: promoter (281 peaks within 1,000 bp of the transcription start site), enhancer (5,821 peaks overlapping enhancers determined by ROSE using the H3K27ac ChIP-seq results), and others (5,511 peaks not at promoters or that do not overlap with enhancers).
- (B) Composite plot of FOXC1, H3K27ac, H3K4me3, and H3K27me3 ChIP-seq signal intensity over 11,613 FOXC1 binding sites, showing that FOXC1 peaks overlapped with H3K27ac signals.
- (C) IGV tracks of the *SMAD3* locus (left) and qRT-PCR quantification showing that *SMAD3* mRNA level (right) increased after FOXC1 knockdown.
- (D) IGV tracks at the *TGFBI* locus (left), showing multiple FOXC1 peaks and the corresponding reduction of H3K27ac signals, as well as qRT-PCR quantification (right) of decreased expression of this gene.
- (E) A table presenting the HOMER motif analysis results using FOXC1 ChIP-seq peaks. The top four *de novo* motif categories and the best-matched TFs representing that motif are shown.
- (F) Gene ontology pathway analysis of genes associated with enhancers bound by FOXC1 in T265 cells.
- (G) Heatmap showing the transcriptional alterations of direct FOXC1-targeted genes upon CRISPR-Cas9-mediated knockout of FOXC1 in T265 cells.
- (H) Enrichment plot demonstrating that Ras signaling genes (De Raedt et al., 2014) were inhibited by FOXC1 knockout in T265 cells. NES, normalized enrichment score.



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transcriptional activation of the downstream interferon genes. In addition, inhibition of Ras signaling by MEK inhibitors (MEKis) has previously been shown to induce STAT signaling (Franklin et al., 2020), and phosphorylated ERK2 can act as a TF whose transcriptional activity can be modulated by MEKis (Tee et al., 2014; Yohe et al., 2018). To test this, we treated T265 cells with the MEKi trametinib, and assessed the induction of these immune-modulatory TFs. Importantly, treatment with the MEKi recapitulated the upregulation of multiple STAT and IRF family members (Figure 4H). Finally, treatment of three PRC2-deficient cell lines with a MEKi resulted in the upregulation of HLA-A, -B, and -C on the cell surface (Figure 4I). In summary, PRC2 restoration caused upregulation of immune genes, and this effect was mediated by inhibition of Ras signaling (Figure S4G).

Single-cell sequencing of PRC2-deficient MPNSTs reveals the cellular heterogeneity within primary and metastatic MPNSTs

By restoring PRC2 in MPNST cells, we identified the aberrant activation of SE-driven TFs and inactivation of immune-related genes caused by the loss of PRC2 in MPNST cells. To correlate the results obtained in cell culture systems with primary human tumors, we performed scRNA-seq on a PRC2-deficient, high-grade MPNST that was sampled at multiple time points through the patient's clinical course (diagnostic needle biopsy, resection of the primary tumor, and resection of a metastatic lung tumor; Figure 5A). After quality control, scRNA-seq data for 56,272 cells were available for downstream analysis, including 20,469 cells from the primary MPNST (9,642 and 10,827 cells from the core needle biopsy and primary resection, respectively) and 35,803 from the metastatic resection. Based on the transcription profile, the cells were separated into 18 distinct clusters (Figure 5B), which were annotated based on the expression of canonical markers (Table S4). These clusters broadly included tumor cells, stromal cells, and immune cells (Figure 5C). There was a notable increase in the tumor cell compartment and contraction of the stromal and immune compartments in the metastatic lesion compared with the primary lesion (Figure 5C and Table S4).

To ensure the accurate identification of the malignant cells within the heterogeneous tumor, copy number variation (CNV) was profiled using single-cell whole-genome profiling (scCNV) in 1,270 cells from the primary tumor. Clustering of the cells based on CNV profiles demonstrated a subgroup of 226 cells that were distinct (Figure S5A). Given that the patient had a

known germline microdeletion at the *NF1* locus, all cells in the primary MPNST exhibited loss of one allele around the *NF1* gene, while a subgroup of cells demonstrated loss of both alleles (Figure S5B). To link scCNV-profiled cells with scRNA-seq profiling, we inferred copy number changes using the scRNA-seq data compared with the immune component of this dataset (Figure S5C). The MPNST cells profiled for scCNV matched precisely with the CNV pattern predicted by the scRNA-seq, indicating proper classification of the malignant cells. Gain of chromosome 8, which has been proposed as an early or founding genomic change in MPNST (Dehner et al., 2021), was a common unique feature of the identified tumor cells (Figure S5D), and was identified by both scRNA-seq and scCNV.

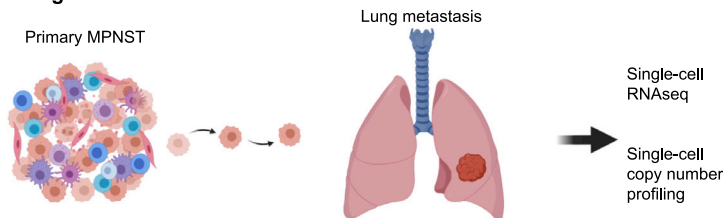
Using the scRNA-seq data, we derived marker genes that defined each distinct cell cluster (Figures S6A and S6B; Table S4) within the primary and metastatic MPNSTs. Oncogenic pathway and hallmark gene set enrichment of the marker genes in the malignant cells revealed key oncogenic processes and, strikingly, pathways involved in the epithelial-mesenchymal transition and maintenance of embryonic stem cells (Figure 5D and S6C). Genes involved in these pathways included target genes of FOXC1, such as *PRRX1*, *WIF1*, and *TGFBI*, that play critical roles during fate commitment of stem cells (Soldatov et al., 2019). This observation validated our findings from the inducible model systems that PRC2 loss activates FOXC1 and other key TFs in MPNST cells.

The large number of cells in the tumor cell compartment of the metastatic MPNST allowed us to identify distinct tumor subclusters within that lesion. These subclusters were present in the primary tumor, albeit at lower numbers (Figures 5E and 5F). One subcluster of cells (cluster 7) that dominated the primary tumor was markedly diminished in the metastatic tumor (64.2% versus 0.5% of malignant cells in the primary versus the metastatic MPNST, respectively; Figures 5E and 5F). Interestingly, the marker genes that characterized this cluster were the immune genes, including IFN and antigen presentation genes, that we identified as indirectly upregulated by PRC2 in our inducible cell line system (Figure S6D). By comparing the expression levels of these genes between the primary and the metastatic tumors, we discovered that these genes were significantly more highly expressed in the primary MPNST (Figure S6E). Interestingly, FOXC1 and Ras expression profiles were noted to be expressed universally, with notable increases in the cells taken from the metastatic lesion (Figures 5G and 5H). Overall, our analysis of scCNV and scRNA-seq of the paired primary and metastatic

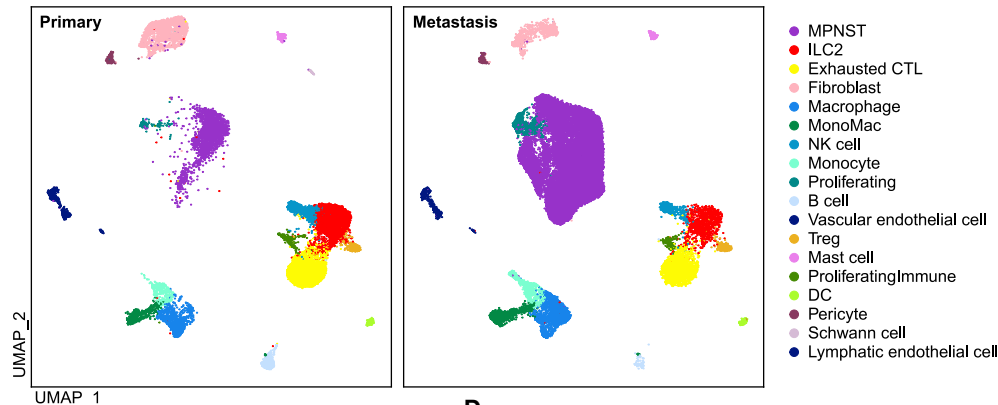
Figure 4. PRC2 restoration indirectly upregulates interferon signaling and antigen presentation

- Network plot showing the connections of upregulated genes upon PRC2 restoration (top four significantly enriched pathways shown). DEGs were derived from comparing the effect of PRC2 restoration to controls in all three PRC2-null MPNST cell lines using RNA-seq (Table S1).
- Quantification of DEGs demonstrating that the genes that were upregulated after PRC2 restoration were not direct PRC2 targets and did not gain H3K27me3 with a functional PRC2.
- Quantification of the PRC2-target DEGs shows that they were not bivalent genes.
- Enrichment plot demonstrating that PRC2 restoration significantly increased genes in the GO pathway "Response to type I interferon".
- Enrichment plot showing that genes associated with the pathway "Antigen presentation folder assembly and peptide loading of class I MHC" were significantly increased upon PRC2 restoration.
- Enrichment plot showing that Ras signaling genes (De Raedt et al., 2014) were inhibited by PRC2 restoration in T265 cells. NES, normalized enrichment score.
- Heatmap of key STAT and IRF transcription factors shows that mRNA expression levels of these transcription factors were increased by PRC2 restoration.
- Heatmap showing the transcriptional activation of STAT and IRF members caused by MEK inhibitor treatment in T265 cells.
- Histogram of flow cytometry result demonstrating the increase in cell-surface expression of MHC1 in three PRC2-deficient cell lines after trametinib treatment.

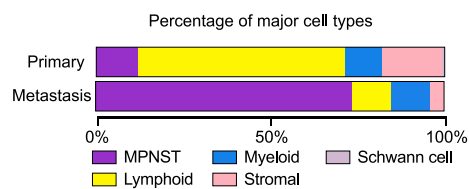
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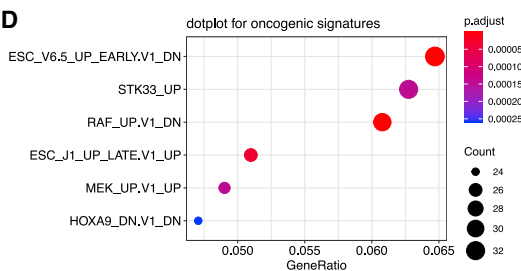
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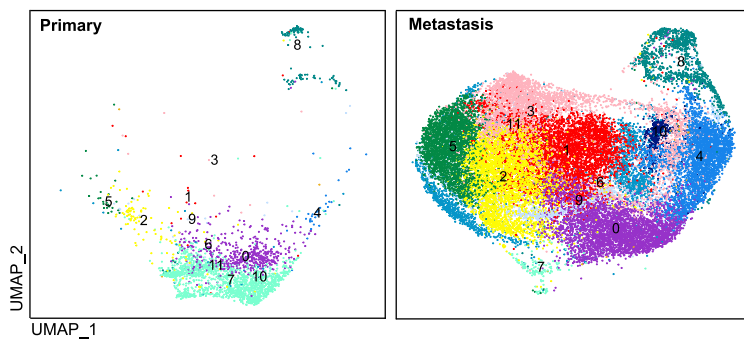
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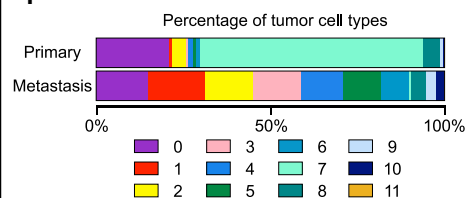
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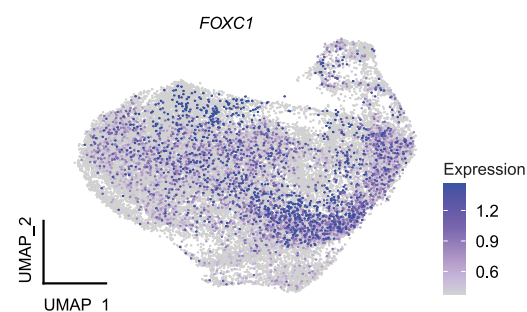
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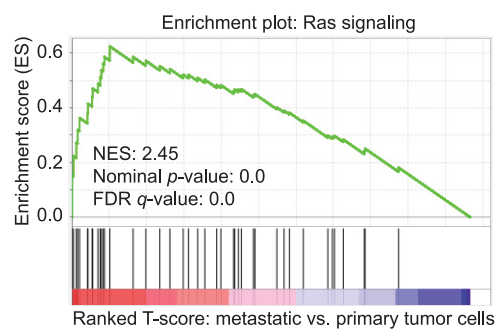
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MPNSTs correlated with the model system findings that PRC2 loss maintains a stem-like oncogenic transcriptional program in the malignant cells and simultaneously diminishes immune presentation of the tumor cell.

Malignant cells in MPNSTs map to a dedifferentiated phenotype

Loss of PRC2 in MPNST results in an upregulation of stem cell signature genes in both the cell model systems and patient tumors, and PRC2 restoration in the PRC2-deficient cells reversed this process. Given these results and the known functions of PRC2 in normal Schwann cell (SC) development (Ma et al., 2015, 2016, 2018), we hypothesized that the MPNST cell might be harnessing a distinct developmental transcription program along the SC trajectory. To evaluate this hypothesis, we used single-cell transcriptomic profiling of mouse tissues (Furlan et al., 2017; Soldatov et al., 2019; Wolbert et al., 2020) at each SC developmental stage (E8.5, E12.5/E13.5, mature) to build a normal SC developmental trajectory. We merged single-cell transcriptomes of normal SCs and their precursors from three individual scRNA-seq studies and computationally integrated them to build a developmental trajectory (Figure 6A). As expected, uniform manifold approximation and projection for dimension reduction (UMAP) clustering identified each subtype of the SC lineage moving from neural crest stem cell (NCSC) to SC precursor (SCP) and branching into the transcriptionally distinct nonmyelinating SC (nmSC) and myelinating SC (mySC). The integrated analysis of these cells depicted a developing SC trajectory with the pseudotimes matching the known chronological development order (Mirsky and Jessen, 1996; Mirsky et al., 1996) (Figure 6B). Highly expressed TFs are the key drivers of this lineage during development and are responsible for cell-fate determination (Hnisz et al., 2013; Saint-André et al., 2016; Whyte et al., 2013). Therefore, we derived marker genes of each cell type based on differential expression analysis and used SCENIC (Aibar et al., 2017) to infer the key TFs characteristic of each developmental stage. Marker genes of each cell type accurately distinguished among cell types (Figure S7A; Table S5). In addition, critical TFs known to distinguish particular cell subtypes were correctly placed on the developmental trajectory (Figure 6C), such as the expression of EGR2 and AP1 family TFs (e.g., FOS, JUNB) in the mySC (Stolt and Wegner, 2016). Strikingly, the previously identified PRC2-regulated SE-driven TFs, PRRX1/2 and FOXC1, were among the unique identifiers of the mesenchyme NCSC. Therefore, we hypothesized that the PRC2-deficient MPNST cell was harnessing transcriptional circuits of the mesenchyme NCSC. To test this hypothesis in an unbiased way, we used AUCell (Aibar

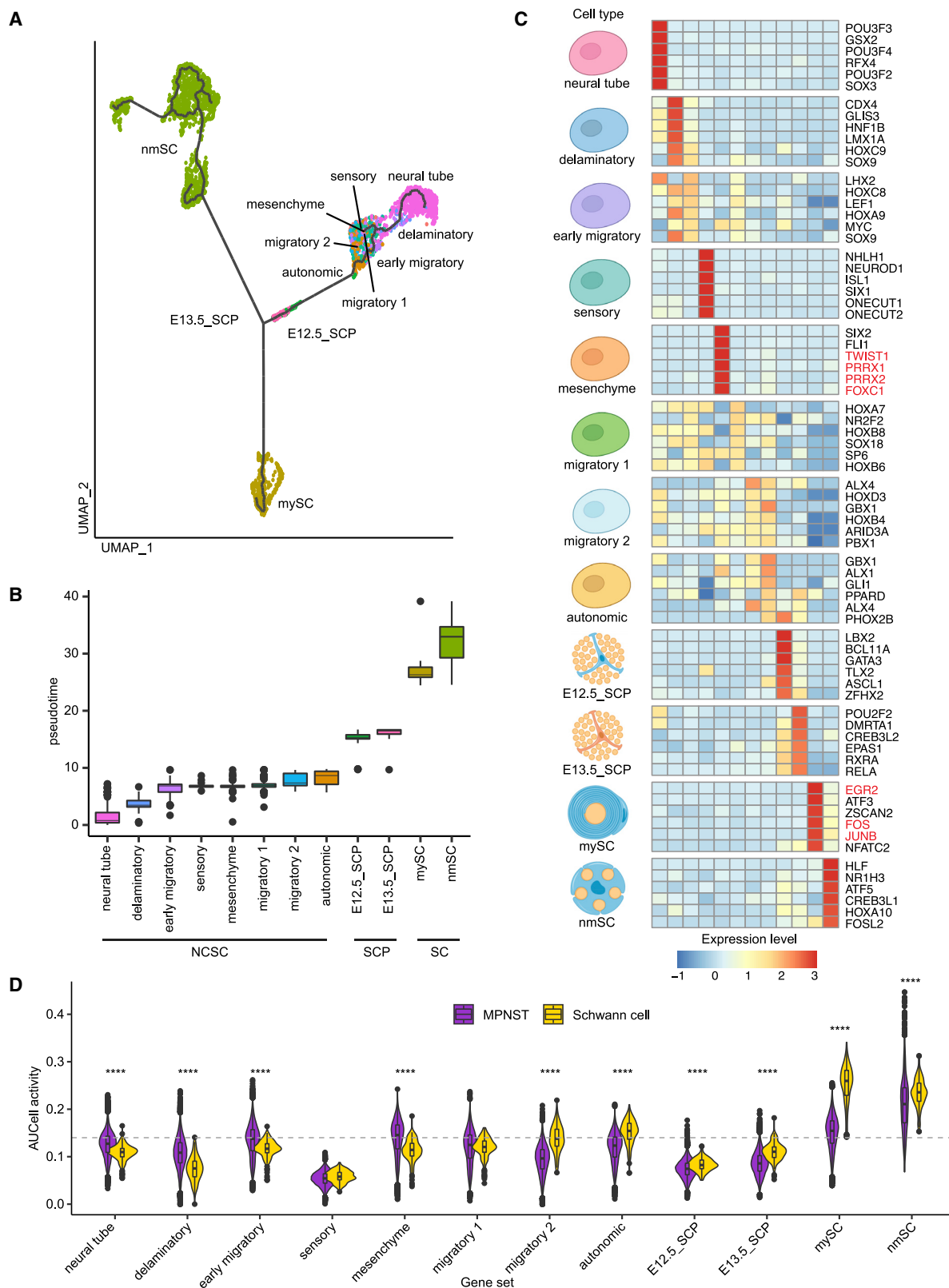
et al., 2017) to compute the expression of marker genes, derived from cells in the normal SC developmental trajectory, in malignant cells and normal SCs identified in the human MPNST scRNA-seq data. As predicted, normal SCs from the primary MPNST exhibited the highest similarity to mature mySC, followed by nmSC (Figure 6D; Table S5). Malignant MPNST cells highly expressed marker genes of nmSC but showed lower expression of marker genes of mySC while also upregulating the expression of marker genes characteristic of mesenchyme NCSC ($p < 0.0001$) and early migratory cells (Figure 6D). Overall, these results suggest that malignant MPNST cells activated a transcriptional program that is characteristic of the mesenchyme NCSC along the normal SC developmental trajectory.

PRC2-regulated TFs establish and reinforce the oncogenic program of MPNST

Next, we compared the transcriptional programs of the malignant cells (27,796 cells from primary and metastasized MPNSTs) with the benign SCs (161 cells from the primary MPNST) of the MPNST and identified genes that were uniquely expressed in the malignant cells (838 genes) and benign SCs (1,493 genes, Table S6). The transcriptomic differences established by these two groups of marker genes could distinctly separate the benign cells from the malignant cells (Figure 7A). Next, we compared these genes with the differentially expressed genes in our PRC2 inducible MPNST cell lines (Figure 1A) and CRISPR-Cas9 FOXC1 or HOXB8 knockout cells (Figures 2C and 2D). Genes downregulated by PRC2 restoration or FOXC1 or HOXB8 loss were significantly enriched among the markers of malignant cells, while genes upregulated by these manipulations were enriched among the SC markers (Figure 7B). The average expression levels of TFs that were downregulated with PRC2 restoration (155 TFs, Table S6) were distinctly highly expressed in the malignant cells (Figure 7C, left). In addition, the directly PRC2-targeted TFs (81 TFs, Table S6) distinguished the malignant cells from all other cells within the patient MPNST (Figure 7C, right). Indeed, the expression levels of SE-driven TFs (40 TFs shown in Figure 2A) were significantly higher compared with other TFs (Figure 7D), indicating that the transcriptional network formed by these highly expressed, SE-driven TFs participates in the oncogenic program that drives the human MPNST. To validate these findings in a broader panel of tumors, we interrogated FOXC1, all PRC2-downregulated TFs, and directly PRC2-targeted TFs in a panel of eight low-passage MPNST patient-derived xenograft models (Figures S7B, S7C, and S7D). These PRC2-regulated TFs were universally expressed by MPNST cells.

Figure 5. Single-cell sequencing reveals intratumoral heterogeneity of human malignant peripheral nerve sheath tumor

- Graphic illustration of the experimental approach for sampling the patient malignant peripheral nerve sheath tumors (MPNSTs).
- Uniform manifold approximation and projection for dimension reduction (UMAP) plot showing the distinct cell types detected in different stages of human MPNST using scRNA-seq.
- Bar graph demonstrating the percentage of major cell types detected in human MPNSTs (Table S4).
- Dot plot of the top six oncogenic pathways enriched among the marker genes of MPNST malignant cell.
- UMAP plot demonstrating the reclustering of malignant cells of human MPNST.
- Bar graph showing the quantification of the number of cells within each cluster of the reclustered malignant cells. Metastasized MPNST lost cluster 7 tumor cells.
- Feature plot of FOXC1 expression in the primary and metastatic tumor cells.
- Gene set enrichment plot demonstrating that Ras signaling was enriched in the metastatic tumor cells.



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To refine the discovered TF circuit, motif analysis was performed to identify potential transcriptional regulators of the malignant marker genes in MPNST. First, we scanned the promoters and enhancers near these genes as defined by the H3K27ac peaks in the inducible MPNST cell lines. This analysis demonstrated that known motifs of the SE-driven TFs were positively enriched in the identified promoters (Figure 7E, left) and enhancers (Figure 7E, right). Strikingly, FOXC1 binding sites were overrepresented in promoters and enhancers of malignant marker genes (Figure 7E). Motifs for TFs directly regulated by FOXC1 (Figure 3F), such as *GLI3*, *TWIST1*, and *PRRX1/2*, were also positively enriched in the promoters and enhancers of these marker genes. The high expression of these FOXC1 directly regulated genes was unique to the malignant cells of the patient MPNST sample and these genes were not found to be expressed in the benign SCs (Figure S7E). To confirm this observation in an independent patient, we performed scRNA-seq from a second PRC2-deficient human MPNST and performed clustering analysis (Figure 7F). Again, FOXC1 was highly expressed and uniquely defined the malignant cells of this patient's tumor (Figure 7G). Mirroring our previous observations, the average expression levels of the defined SE-driven TFs were again significantly higher compared with the other TFs (Figure 7H). Finally, results of the motif enrichment analysis showed that the FOXC1 motif was significantly positively enriched in the enhancers of the marker genes of the malignant cells of this tumor (Figure 7I), indicating its potential function in sustaining the oncogenic program of MPNST. Overall, these results suggest that PRC2 loss and subsequent activation of FOXC1 serves a critical transcriptional regulatory role in driving the oncogenic program of human MPNST.

DISCUSSION

Loss or dysregulation of functional PRC2 is a common oncogenic lesion in human cancer (Comet et al., 2016), and deletions in PRC2 have been shown to be enriched in chemotherapy-resistant Ras mutant tumors (Guerra et al., 2020). Combining inducible models of PRC2 reconstitution and single-cell profiles from clinical specimens, we provide mechanistic details of how the genetic loss of PRC2 activates developmentally specific TFs and enforces stem-like properties in the MPNST cell while dampening the immune presentation of the cell. Specifically, our studies revealed that loss of PRC2 results in direct loss of H3K27me3 and gain of H2K27ac at bivalent, developmentally regulated genes, including *FOXC1* and *HOXB8*. Mechanistically, these changes activate a gene program characteristic of a mesenchymal stem cell and concurrently suppress Ras-mediated, STAT/IRF-driven programs that are critical for immune surveillance. These findings have important implications for how

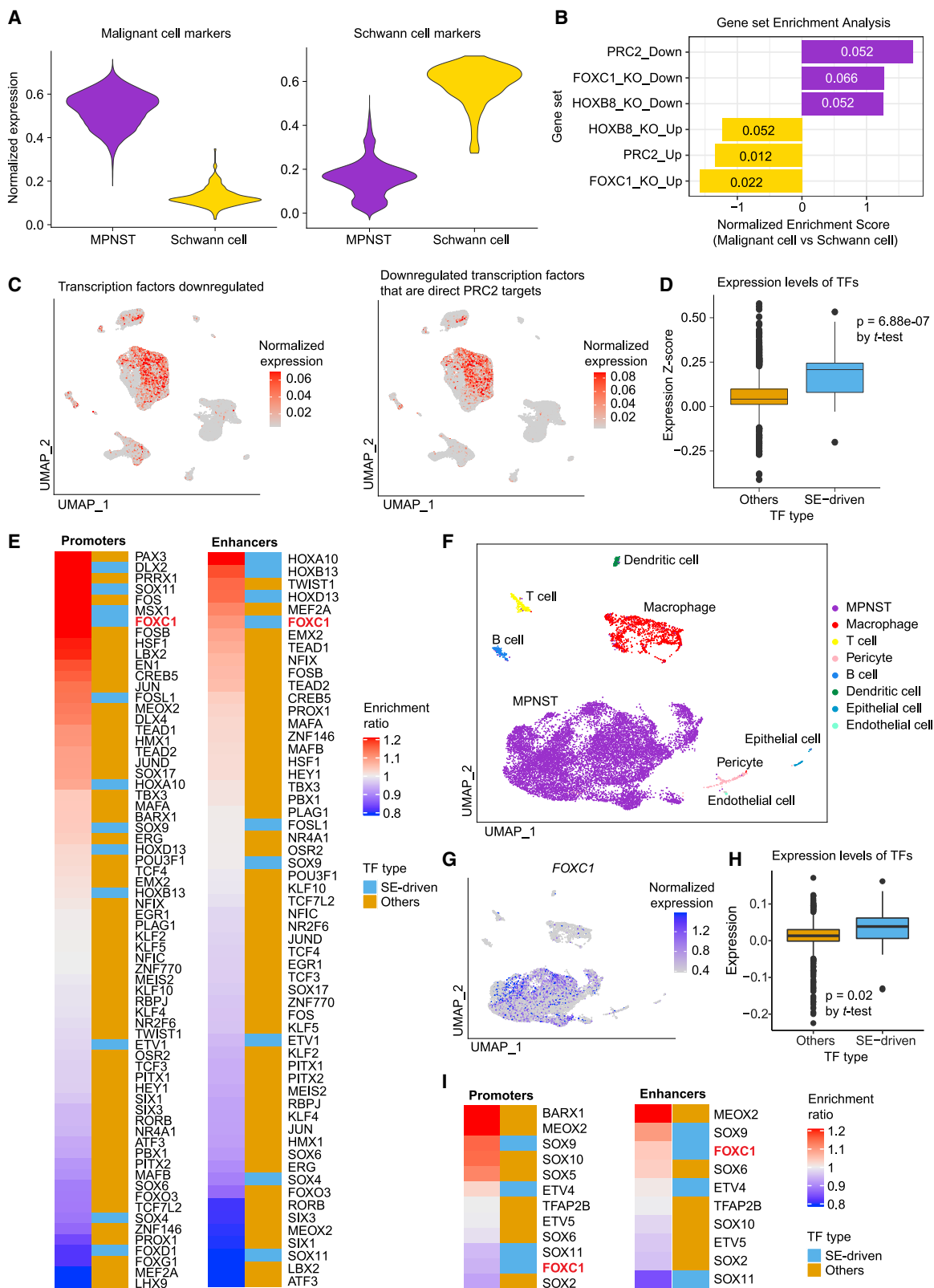
future therapeutic strategies might be employed to target these highly refractory tumors.

Our study defined a group of core TFs, including FOXC1 and HOXB8, that are upregulated as a result of loss of H3K27me3 and gain of H3K27ac in the MPNST cell. Enhancers marked by extensive stretches of H3K27ac determine cell fate and identity by driving the expression of master TFs (Whyte et al., 2013). Multiple studies of diverse cancer subtypes have demonstrated the critical role that these cell-type-specific SEs and TFs play in tumorigenesis (Decaestecker et al., 2018; Durbin et al., 2018; Ran et al., 2018). Our work nominated FOXC1 as a PRC2-dependent switch that is activated in the malignant transformation of the MPNST cell. FOXC1 is known to activate transcription of downstream targets (Hannenhalli and Kaestner, 2009; Jackson et al., 2010; Pierrou et al., 1994) and has previously been shown to regulate proliferation and the epithelial-to-mesenchymal transition that defines a clinically aggressive subtype of breast cancer (Ray et al., 2010; Taube et al., 2010). Interestingly, in acute myeloid leukemias (AMLs), 20% of tumors have FOXC1 overexpression due to regional loss of H3K27me3 at the FOXC1 promoter, which is correlated with co-existing overexpression of members of the HOX TF family (Somerville et al., 2015), mirroring our observations in MPNST. In addition to PRC2-mediated regulation, transcription of FOXC1 is induced by activated mitogen-activated protein kinase (MAPK) signaling (Cauchy et al., 2015). In this context, FOXC1 activates an abnormal regulatory network defined by its downstream target genes (Assi et al., 2019). The potential interplay of FOXC1 with hyperactivated Ras signaling is further evidenced by the presence of an ERK dependent phosphorylation of FOXC1 that improves its protein stability and transcriptional activities (Berry et al., 2006). Further mechanistic understanding of the cross talk between Ras signaling and FOXC1 in the context of the NF1-deficient MPNST cell could further define FOXC1's role as a central transcriptional node in Ras-driven malignancies. While TFs remain in the "undruggable" category, the improvement of small-molecule targeting of transcription (Struntz et al., 2019) and the development of proteolysis targeting chimeric (PROTAC) technology (Bushweller, 2019) provide future potential therapeutic avenues to target these vulnerabilities in MPNST.

Our findings highlight that the MPNST cell transcriptional program mirrors gene programs characteristic of neural-crest-derived mesenchymal cells. SCs from the dorsal root ganglia are known as the cell of origin of NF1-associated plexiform neurofibromas (PNFs) (Chen et al., 2014) and these neoplastic *NF1*^{-/-} SCs undergo malignant transformation with the loss of *CDKN2A* and PRC2 to give rise to MPNSTs (Kim et al., 2017; Zhang et al., 2020). Our analysis discovered that the MPNST cell accomplishes this by corrupting the SC-specific transcriptional regulatory network pioneered by stage-specific TFs (Ma

Figure 6. MPNST malignant cells demonstrate dedifferentiated phenotype regulated by PRC2

- (A) UMAP plot of Schwann cell (SC) development from various cell types within the neural crest (NCSC) through SC precursors (SCP) to mature myelinating SC (mySC) and nonmyelinating SC (nmSC). Analysis was done using publicly available scRNA-seq data (see STAR Methods for details).
(B) Boxplot demonstrating the pseudotime of cells along the SC developmental trajectory.
(C) Transcriptional activity of SC development demonstrated by the most active transcription factors at each developmental stage.
(D) Comparison of AUCell activities of malignant MPNST cells with normal SCs using the marker genes of developing SCs (Table S5) at various developmental stages. Malignant cells demonstrated high activity of nmSC, mySC, and mesenchyme NCSC; ****p < 0.0001.



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et al., 2018; Stolt and Wegner, 2016) that are driven by H3K27ac-marked enhancers (Ghislain and Charnay, 2006; Jagalur et al., 2011; Reiprich et al., 2010). Our integrated scRNA-seq analysis enabled us to compare the MPNST cell across the entire trajectory of normal SC development. Our observation that MPNST cells transcriptionally mirror mesenchymal cells is consistent with the observation of stem-cell-like features of MPNST reported by others (Spyra et al., 2011; Sun et al., 2021). In addition, it is interesting to note FOXC1's role in development and neural crest specification (Rice et al., 2003; Sun et al., 2013; Tribulo et al., 2003). Indeed, FOXC1 is transiently expressed in the mesenchymal NCSCs during the early phase of SC development and thereafter is properly repressed by PRC2-mediated H3K27me3 as the cells commits to an SC lineage (Soldatov et al., 2019). The understanding of MPNST in the context of embryonic development is reminiscent of other tumors of neural crest origin, such as neuroblastoma (Dong et al., 2020; Jansky et al., 2021; Kameneva et al., 2021). Future efforts could be designed to epigenetically drive the MPNST cell toward a more differentiated phenotype that would eliminate the self-renewal capacity and migratory nature of these cells (de Thé, 2018).

Finally, the incorporation of single-cell sequencing of clinical samples allowed us to interrogate the cancer cell-immune cell cross talk in MPNSTs. Our results align well with the reports from elegant proteomic studies that found decreased interferon signaling and major histocompatibility complex (MHC) class I-mediated antigen presentation in PRC2-deficient MPNSTs (Wojcik et al., 2019). Interestingly, PRC2 regulation of genes involved in immune surveillance has previously been reported in PRC2-competent cancers. In this context, abnormally increased PRC2 activity was directly responsible for the transcriptional silencing of MHC class I-mediated antigen presentation (Burr et al., 2019). In contrast, in PRC2-deficient MPNSTs, these same MHC class I genes were not direct PRC2 targets and did not gain H3K27me3 when a functional PRC2 was restored. Instead, we find that PRC2 restoration directly downregulates hyperactive Ras signaling, which dampens STAT/IRF signaling and results in the indirect transcriptional activation of the immune-related genes. These results indicate that, while neither MEK inhibition nor immunotherapy alone would be successful, combination strategies designed to dampen hyperacti-

vated Ras signaling while activating the immune system may be beneficial in the treatment of MPNST. Excitingly, clinical trials designed to test these combination therapeutic strategies are in development.

Limitations of the study

We report in this study that PRC2 restoration caused indirect transcriptional activation of genes in the type I interferon signaling and antigen presentation pathways. This observation is consistent with a previous study, in which Wojcik et al. found that PRC2 loss was associated with reduction of these immune genes in patient MPNST samples (Wojcik et al., 2019). Using engineered MPNST cell lines coupled with flow cytometry, we revealed modest increases in cell-surface expression of HLA class I molecules after PRC2 restoration. This is likely due to the limitation of the *in vitro* model systems used in this study; the profound dampening of antigen presentation and interferon signaling by PRC2 inactivation in MPNST patient samples is likely further influenced by the complex tumor microenvironment, which cannot be modeled or tested in cell lines. Future studies using immune-competent animal models of SC-specific PRC2 inactivation will be useful to fully evaluate these changes. Another key limitation of our study is the inclusion of a small number of clinical samples available for transcriptomic profiling. Additional studies of larger cohorts of patients will be required to validate our observations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Cell lines and culture conditions
 - Patients
- METHOD DETAILS
 - Construction of the inducible expression vectors

Figure 7. Activation of PRC2-regulated transcription factors drives the malignant program in human MPNST samples

- (A) Violin plots of malignant cell marker genes (left) and Schwann cell (SC) marker genes (right) in the MPNST scRNA-seq dataset. Average expression of these marker genes in 27,796 malignant cells and 161 benign SCs is shown.
- (B) Bar plot showing the results of pathway enrichment analysis among malignant cell and SC marker genes. Genes that were downregulated by HOXB8 or FOXC1 knockout or PRC2 restoration were significantly positively enriched among the malignant cell markers; p_{adj} is shown.
- (C) UMAP plots demonstrating that the high expression of all PRC2-regulated TFs (left) and direct PRC2 targets among these TFs (right) was unique to the malignant MPNST cells.
- (D) Boxplot of the expression levels of all TFs in malignant cells of human MPNST. TFs that had at least one assigned super enhancer (SE-driven) were expressed at a significantly higher level than the others.
- (E) Heatmaps of motif enrichment ratios of the TFs at the promoters (left) and in the enhancers (right) of marker genes of the MPNST malignant cells. The TFs are ranked from the highest to the lowest ratios. Blue, SE-driven TF; yellow, other TFs.
- (F) UMAP plot of an MPNST single-cell dataset from another patient, featuring transcriptomic profiling of 14,967 cells.
- (G) UMAP plot demonstrating that high expression levels of FOXC1 in the malignant cells (12,721 cells) could distinctly separate them from the tumor micro-environment.
- (H) Boxplot of expression levels of all TFs in malignant cells of the second human MPNST. TFs that had at least one assigned super enhancer (SE-driven) were expressed at a significantly higher level compared with the other TFs.
- (I) Heatmap of motif enrichment ratios of the TFs at the promoters (left) and in the enhancers (right) of marker genes of the second MPNST's malignant cells, ranked from the highest to the lowest ratio. Blue, SE-driven TF; yellow, other TFs.

- Protein isolation, histone extraction and western blotting analysis
- Co-immunoprecipitation
- RNAseq
- Quantitative real-time PCR (qRT-PCR)
- ChIP-seq
- CUT&RUN and sequencing
- ATAC-seq
- Antibodies
- Transient transfection
- Customized siRNA library screening
- CRISPR-Cas9 mediated gene knockout
- Soft agar clonogenic assay
- Flow cytometry
- Single-cell RNAseq
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
 - RNAseq data analysis
 - ChIP-seq and CUT&RUN data analysis
 - Single-cell RNAseq data analysis
 - Single-cell whole genome sequencing and data processing
 - Construction of a normal schwann cell developmental trajectory
 - Motif analysis in promoter and enhancers
 - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.111363>.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.Z. and J.F.S.; methodology, X.Z.; software, X.Z., V.G., and H.L.; formal analysis, X.Z., H.E.L., V.G., Z.L., and H.L.; investigation, X.Z., H.E.L., Z.L., H.M.J., and C.P.J.; resources, X.Z., Z.L., M.E.Y., P.C.,

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FOXC1	Cell Signaling Technology	Cat# 8758S; RRID: AB_2797657
FOXC1	Invitrogen	Cat# PA1-807; RRID: AB_2247078
EZH2	Cell Signaling Technology	Cat# 5246S; RRID: AB_10694683
SUZ12	Cell Signaling Technology	Cat# 3737S; RRID: AB_2196850
EED	Abcam	Cat# ab4469; RRID: AB_2262066
H3K27me3	Cell Signaling Technology	Cat# 9733S; RRID: AB_2616029
H3K27me3	Millipore	Cat# 07-449; RRID: AB_310624
H3K4me3	Cell Signaling Technology	Cat# 9751S; RRID: AB_2616028
H3K27ac	Abcam	Cat# ab4729; RRID: AB_2118291
pERK	Cell Signaling Technology	Cat# 9101S; RRID: AB_331646
ERK	Cell Signaling Technology	Cat# 9102S; RRID: AB_330744
IgG isotype control	Cell Signaling Technology	Cat# 3900S; RRID: AB_1550038
VINCULIN	Cell Signaling Technology	Cat# 13901S; RRID: AB_2728768
B-ACTIN	Cell Signaling Technology	Cat# 3700S; RRID: AB_2242334
CAS9	Cell Signaling Technology	Cat# 14697S; RRID: AB_2750916
IgG HRP conjugated secondary	Cell Signaling Technology	Cat# 7076; RRID: AB_330924
IgG HRP conjugated secondary	Cell Signaling Technology	Cat# 7074; RRID: AB_2099233
HOXB8	Santa Cruz	Cat# sc-517156
PRRX2	Invitrogen	Cat# PA5-3719S; RRID: AB_2553954
IgG HRP light chain specific secondary	Jackson Laboratory	Cat# 221-002-171
HLA-A,B,C	Biolegend	Cat# 311406; RRID: AB_314875
IgG2a, K Isotype Ctrl	Biolegend	Cat# 400214; RRID: AB_2800438
BST2	Cell Signaling Technology	Cat# 19277S; RRID: AB_2798815
B2M	Cell Signaling Technology	Cat# 12851S
IFITM1	Cell Signaling Technology	Cat# 13126S; RRID: AB_2798126
IFITM2	Cell Signaling Technology	Cat# 13530S; RRID: AB_2798248
IFITM3	Cell Signaling Technology	Cat# 59212S; RRID: AB_2799561
Stat1-6	Cell Signaling Technology	Cat# 93130T
Bacterial and virus strains		
Edit-R Inducible Lentiviral Cas9	Dharmacon	Cat# VCAS11227
Chemicals, peptides, and recombinant proteins		
DMEM Medium	Thermo Fisher	Cat# 11995081
Fetal Bovine Serum	Bio-technie	Cat# S11550
Penicillin-Streptomycin	Thermo Fisher	Cat# 15070063
RPMI Medium	Thermo Fisher	Cat# 11879020
Puromycin	Thermo Fisher	Cat# A1113803
Doxycycline	Sigma Aldrich	Cat# D9891
10x Cell Lysis Buffer	Cell Signaling Technology	Cat# 9803
Protease Inhibitor Cocktail	Thermo Fisher	Cat# 78430
4x LDS Sample Loading Buffer	Invitrogen	Cat# NP0008
Skim Milk Powder	MP Biomedicals	Cat# 902887
SuperSignal West Pico Chemiluminescent substrate	Thermo Fisher	Cat# 34080

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
SuperSignal West Femto Chemiluminescent substrate	Thermo Fisher	Cat# 34094
Clarity Max Western ECL Substrate	Bio-Rad	Cat# 1705062
Tween Ultrapure	Affymetrix	CAS# 9005-64-5
Tris Buffered Saline, 10X	Corning	REF# 46-012-CM
Lipofectamine RNAiMAX	Invitrogen	Cat# 13778-150
SPRiselect reagent	Beckman	Cat# B23317
Opti-MEM	Gibco™	Cat# 31985062
CellTiter 96 AQueous One Solution	Promega	Cat# G3582
Blasticidin	Invitrogen	Cat# A1113902
Puromycin	Invitrogen	Cat# A1113802
Anti-Fc receptor	Biolegend	Cat# 422301
Fixable Viability Dye eFluor™ 506	Invitrogen	Cat# 65-0866-14
collagenase I	STEMCELL Technologies	Cat# 07416
dispace II	MilliporeSigma	Cat# D4693-1G
DNase I	Invitrogen	Cat# 18047019
AO/PI viability dye	Nexcelom	Cat# CS2-0106-5ML

Critical commercial assays

Essen IncuCyte FLR	Essen BioScience	Cat# 4515
Bradford Assay Dye	Bio-Rad	Cat# 5000006
Total Histone Extraction Kit	Epigentek	OP-0006-100
Dynabeads M-280 Sheep anti-Rabbit IgG Magnetic Beads	Invitrogen	Cat# 11203D
RNeasy Mini Kit	Qiagen	Cat# 74004
TruSeq Stranded Library Preparation Kit	Illumina	Cat# 20020594
ChIP-IT High Sensitivity kit	Active Motif	Cat# 53040
TruSeq ChIP Library Prep Kit	Illumina	Cat# IP-202-1012
Zymo DNA Clean and Concentrator-5 Kit	Zymo Research	Cat# D4014
NexteraDNA Library Prep kit	Illumina	Cat# FC-131-1024
High-Capacity RNA-to-cDNA kit	ThermoFisher	Cat# 4387406
CUT&RUN assay kit	Cell Signaling Technology	Cat# 86652
SimpleChIP ChIP-seq DNA library Prep Kit	Cell Signaling Technology	Cat# 56795
Multiplex Oligos for Illumina® (Dual Index Primers)	Cell Signaling Technology	Cat# 47538

Deposited data

Next generation sequencing data generated for this study	This study	GSE183309
ATAC-seq data generated	This study	GSE183305
ChIP-seq data generated	This study	GSE183306
RNAseq data generated	This study	GSE183307
Single-cell RNAseq data generated	This study	GSE183308
CUT&RUN data generated	This study	GSE202671
E9.5 neural crest cell single-cell sequencing data	Soldatov et al. (2019)	GSE129114
E12.5/E13.5 adrenal medulla cell single-cell sequencing data	Furlan et al. (2017)	GSE150150
Mature Schwann cell single-cell sequencing data	Wolbert et al. (2020)	GSE142541

Experimental models: Cell lines

T265 cell line	Laboratory of Dr. Karlyne Reilly	RRID: CVCL_S805
S462 cell line	Laboratory of Dr. Karlyne Reilly	RRID: CVCL_1Y70
SNF96.2 cell line	Laboratory of Dr. Karlyne Reilly	RRID: CVCL_K281

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
SNF02.2 cell line	ATCC	Cat# CRL-2885; RRID: CVCL_K280
STS-26T cell line	Laboratory of Dr. Steven Porcelli	RRID: CVCL_8917
MPNST724 cell line	Laboratory of Dr. Nancy Ratner	RRID: CVCL_AU20
ST88-14 cell line	Laboratory of Dr. Nancy Ratner	RRID: CVCL_8916
PT2002 cell line	Johns Hopkins NF1 Biospecimen Repository	N/A
PT2031 cell line	Johns Hopkins NF1 Biospecimen Repository	N/A
PDX cell line	Laboratory of Dr. Rosandra Kaplan	N/A
HEK293 cells	Takara Bio	Cat# 632180
Oligonucleotides		
Negative control siRNA	Qiagen	Cat# 1027281
siFOXC1-1	Qiagen	Cat# SI00026138
siFOXC1-5	Qiagen	Cat# SI03037713
siHOXB8-4	Qiagen	Cat# SI00440685
siHOXB8-6	Qiagen	Cat# SI04311916
Recombinant DNA		
ORF of human SUZ12	GenScript	Cat# NM_015355
PLVX-TetOne-Puro vector	Takara Bio	Cat# 631849
pLentiGuide-Puro vector	GenScript	
Software and algorithms		
Quantity One Software	Bio-Rad	https://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software
BWA	Li and Durbin, 2009	https://github.com/lh3/bwa
DESeq2	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
macs2 v2.2.6	Zhang et al., 2008	https://github.com/mac3-project/MACS
DeepTools	Ramírez et al., 2016	https://deeptools.readthedocs.io/en/develop/
IGV genome browser	Robinson et al., 2011	https://software.broadinstitute.org/software/igv/
STAR	Dobin et al., 2013	https://github.com/alexdobin/STAR
RSEM	Li and Dewey, 2011	https://github.com/deweylab/RSEM
R package ComplexHeatmap	Gu et al., 2016	https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html
BEDOPS	Neph et al., 2012	https://bedops.readthedocs.io/en/latest/
Trimmomatic	Bolger et al., 2014	http://www.usadellab.org/cms/?page=trimmomatic
BEDTools		https://bedtools.readthedocs.io/en/latest/index.html
HOMER	Heinz et al., 2010	http://homer.ucsd.edu/homer/download.html
Rank Ordering of Super-Enhancer (ROSE)	Lovén et al., 2013	https://bitbucket.org/young_computation/rose
FlowJo software	BD	https://www.flowjo.com/solutions/flowjo/downloads
Loupe scDNA Browser	10X Genomics	https://support.10xgenomics.com/single-cell-dna/software/downloads/latest
Euler algorithm in TFBSTools	Tan and Lenhard, 2016	https://bioconductor.org/packages/release/bioc/html/TFBSTools.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NGSplot	Shen et al., 2014	https://github.com/shenlab-sinai/ngsplot
Cell Ranger	10X Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest
Seurat v4.0	Butler et al., 2018; Stuart et al., 2019	https://satijalab.org/seurat/
inferCNV	inferCNV of the Trinity CTAT Project	https://github.com/broadinstitute/inferCNV
ReactomePA	Yu and He, 2016	https://bioconductor.org/packages/release/bioc/html/ReactomePA.html
GraphPad Prism	GraphPad	N/A
Biorender	Biorender	https://biorender.com
HOCOMOCO v11	Kulakovskiy et al., 2016	https://hocomoco11.autosome.org
JASPER 2020	Fornes et al., 2020	https://jaspar.genereg.net
SCENIC	Aibar et al., 2017	https://scenic.aertslab.org
Monocle3	Cao et al., 2019; Trapnell et al., 2014	https://cole-trapnell-lab.github.io/monocle3/

Other

In-Fusion HD	Takara Bio	Cat# 639643
SpectraMax M3 microplate reader	Molecular Devices	Cat# 89429-536
Trans-Blot Turbo Transfer system	Bio-Rad	N/A
ChemiDoc	Bio-Rad	N/A
Agilent 2100 Bioanalyzer	Agilent	N/A
HiSeq4000 sequencer	Illumina	N/A
EpiShear Probe Sonicator	Active Motif	N/A
Thermomixer	Eppendorf	N/A
Illumina NextSeq machine	Illumina	N/A
Incucyte S3 live cell analysis system	Essen BioScience	N/A
Nikon Eclipse TE300 microscope	Nikon	N/A
LSRFortessa	BD	N/A
GentleMACS dissociator	Miltenyl Biotec	N/A
Cellometer Auto 2000	Nexcelom	N/A
Chromium Controller	10x Genomics	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jack F. Shern (john.shern@nih.gov).

Materials availability

DNA constructs and other research reagents generated by the authors will be distributed upon request to other researchers.

Data and code availability

- All datasets used are summarized in the [key resources table](#). Raw and processed next generation sequencing data generated for this study have been deposited in the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) with the accession number GEO: GSE183309. This SuperSeries is composed of the following SubSeries: GSE183305, GSE183306, GSE183307, GSE183308, and GSE202671, containing related ATAC-seq, ChIP-seq, RNAseq, single-cell RNA-seq, and CUT&RUN data, respectively.
- This study did not generate any unique code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and culture conditions

MPNST cell lines, T265, S462 and sNF96.2, were kindly provided by Dr. Karlyne Reilly (Pediatric Oncology Branch, NCI) and were maintained in DMEM10 (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μ g/mL streptomycin). Cell line sNF02.2 was purchased from ATCC (Manassas, VA) and maintained in DMEM10. Sporadic MPNST cell line STS-26T was kindly provided by Dr. Steven Porcelli (Albert Einstein College of Medicine) and maintained in DMEM10. Cell lines MPNST724 and ST88-14 were kindly provided by Dr. Nancy Ratner and maintained in DMEM10. NF1 patient-derived MPNST cell lines PT2002 and PT2031 were requested through The Johns Hopkins NF1 Biospecimen Repository (https://www.hopkinsmedicine.org/kimmel_cancer_center/cancers_we_treat/pediatric_oncology/research_and_clinical_trials/pratilas/nf1_biospecimen_repository.html) and kindly provided by Dr. Christine Pratilas. They were cultured in RPMI10 (RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μ g/mL streptomycin). Finally, patient-derived MPNST cell line PDX was established from a primary sporadic MPNST sample in the laboratory of Dr. Rosandra Kaplan (Pediatric Oncology Branch, NCI) and kindly provided to us to be maintained in DMEM10. Lenti-X HEK293 cells were originally purchased from Takara Bio (Mountain View, CA) and maintained in DMEM10 and kindly provided by Dr. Carol Thiele's laboratory (Pediatric Oncology Branch, NCI). All cell culture was carried out in an incubator at 37°C with 5% CO₂ and routinely tested for mycoplasma contamination. Cell lines sNF02.2, sNF96.2, S462, T265 and STS-26T were characterized by the short tandem repeat (STR) analysis and their signatures matched known profiles. In addition, each cell line was subjected to exome-based sequencing and the FASTQ sequencing files were deposited and available for public use (Synapse). For the cell growth assays, the Essen InCuCyte FLR (Essen BioScience, Ann Arbor, MI) was used to monitor the cell confluency over time.

Patients

Three resected MPNST samples and one surgical biopsy from two NF1 pediatric patients (including one female and one male patient; age less than 18) were analyzed in this study. Informed consent was obtained on NCI IRB approved protocol (NCT01109394; IRB identifier 10C0086) for the tumor samples prospectively collected and included in this work.

METHOD DETAILS

Construction of the inducible expression vectors

Open reading frame (ORF) of human *SUZ12* (NM_015355, GenScript, Piscataway, NJ) was cloned into the pLVX-TetOne-Puro vector (Takara Bio) using In-Fusion HD (Takara Bio), following the manufacturer's manual. MPNST cell lines were infected with lentiviral particles generated using either pLVX-TetOne-Puro-SUZ12 or empty vector (pLVX-TetOne-Puro-EV), followed by puromycin selection (STS-26T: 5 μ g/mL, T265: 1 μ g/mL, ST88-14: 5 μ g/mL, and PT2002: 1 μ g/mL). Various doses of doxycycline (Dox) were used in these cells to induce various levels of SUZ12 expression. To profile the epigenetic alterations caused by PRC2 restoration in these cells, Dox concentration of 0.5 μ g/mL was chosen for the reason that it can induce SUZ12 re-expression to its level shown in a PRC2-WT cell line, STS-26T (Figure S1B). For the Dox dose-dependent growth assay, low and high doses used are listed here: T265 (0.1 μ g/mL and 0.5 μ g/mL, respectively), ST88-14 (0.05 μ g/mL and 0.1 μ g/mL, respectively) and PT2002 (0.02 μ g/mL and 0.05 μ g/mL, respectively). Without further specification, all experiments with a functional PRC2 restored used 0.5 μ g/mL Dox for 5 day. Media with Dox was replenished every two days.

Protein isolation, histone extraction and western blotting analysis

To assess protein levels, samples were harvested from cultured cell lines for immunoblotting analysis. Whole cell protein lysate was collected from lysed cells resuspended in 1x Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1x protease inhibitor cocktail (Thermo Fisher, Waltham, MA). Protein concentration was assessed using Bradford assay dye (Bio-Rad, Hercules, CA) and read in the SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA). Protein samples were then denatured in 1x SDS Sample Loading Buffer (Invitrogen) at 95°C for 5 min and 1.5–7.5 μ g of total protein was separated using an SDS-PAGE system by size via gel electrophoresis. Protein was transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer system (Bio-Rad). The membrane was subsequently blocked with 5% non-fat milk dissolved in 0.1% TBS-tween for 1 h at room temperature followed by incubation with primary antibody overnight at 4°C. The membrane was then washed with 0.1% TBS-tween 4 times for 10 min each time followed by one-hour room temperature secondary antibody incubation. The membrane was developed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher), SuperSignal West Femto chemiluminescent substrate (Thermo Fisher), or Clarity Max Western ECL Substrate (Bio-Rad) to be visualized by chemiluminescence imaging system (ChemiDoc, Bio-Rad). To detect histone proteins, harvested cell pellets were used to isolate total histones using Total Histone Extraction Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instruction. Concentration of histone extract was assessed using Bradford assay, and histone protein was denatured in 1x SDS Sample Loading Buffer before being separated by gel electrophoresis. Detection of histone protein was achieved in a similar process as total protein described above. Protein band intensity was assessed and quantified using the Quantity One software (Bio-Rad).

Co-immunoprecipitation

Primary antibodies against FOXC1, EZH2, SUZ12 and IgG isotype control were incubated overnight with Dynabeads M-280 Sheep anti-Rabbit IgG magnetic beads (Invitrogen) followed with washing using Washing Buffer (50 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.005 Triton X-100, 1 mM EDTA, de-ionized H₂O). Whole cell lysates from cultured cells were incubated with the primary antibody-bound magnetic beads under continuous rotation at 4°C for 4 h. Excessive unbound protein was cleared from the beads by washing. After 5 rounds of washing with Washing Buffer, the co-IP products were eluted from the beads by boiling in 1x SDS Loading Buffer at 99°C for 5 min with shaking at 1000 rpm. Three percent of the whole cell lysate input was loaded alongside co-IP products for gel electrophoresis. Nitrocellulose membrane bound with protein was incubated with the corresponding primary antibodies followed by incubation with the sheep-against rabbit light-chain specific secondary antibody. Developed membrane was imaged using ChemiDoc (Bio-Rad) as described above.

RNAseq

Total RNA was isolated and subjected to transcriptomic profiling from MPNST cells that were constructed with the inducible expression vector after they were treated with or without 0.5 µg/mL of Dox for 5 consecutive days. Total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany), per manufacturer's direction. Quality and quantity of RNA samples were assessed using the Agilent 2100 Bioanalyzer at the Genomics Core of the Center for Cancer Research (CCR) in NIH. Samples with an RNA integrity number that was larger than 9.0 were further processed for library construction and sequencing at the sequencing facility at Frederick National Laboratory for Cancer Research (CCRSF). Strand-specific transcriptomic sequencing library was constructed by using the TruSeq Stranded Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer's instruction. Samples were pooled and sequenced on a HiSeq4000 sequencer for paired-end sequencing with a read length of 150 base pair (bp).

Quantitative real-time PCR (qRT-PCR)

Total RNA was converted to cDNA using the High-Capacity RNA-to-cDNA kit (Thermo Fisher). To measure the relative mRNA levels of *FOXC1*, *SMAD3*, *TGFBI*, and *RUNX2*, quantitative PCR was performed in a 384-well plate using the CFX real-time PCR system (Bio-Rad) as previously described (Zhang et al., 2017). The mRNA level of housekeeping gene *HPRT1* was used as the internal control for normalization.

ChIP-seq

ChIP was performed using the ChIP-IT High Sensitivity kit (Active Motif, Carlsbad, CA), following the manufacturer's manual. Briefly, formaldehyde-(1%, room temperature, 15 min) fixed chromatin was sheared to fragments of 200–1000 bp with the EpiShear Probe Sonicator (Active Motif). T265 cells were sonicated at 30% amplitude, pulse for 20 s on and 40 s off for a total sonication “on” time of 15 min; ST88-14 and PT2002 cells were sonicated at 30% amplitude, pulse for 20 s on and 40 s off for a total sonication “on” time of 16 min. Sheared chromatin was immunoprecipitated with antibodies against H3K27me₃, H3K27ac, H3K4me₃ or FOXC1 overnight at 4°C, followed by agarose protein A/G beads enrichment and DNA purification. To date, genome wide FOXC1 binding has not been profiled. A commercially available FOXC1 antibodies was used as it was tested suitable for ChIP-seq assays by ChIP-western blotting and peak calling. Drosophila chromatin (Active Motif) was added to the ChIP reaction and an antibody against Drosophila-specific histone variant H2Av (Active Motif) was included in the pull down. ChIP DNA library was prepared at the CCRSF using TruSeq ChIP Library Prep Kit (Illumina, cat. IP-202-1012) and sequenced on a NextSeq platform for single-end sequencing with a read length of 76 bp. About 25–30 million base-pairs uniquely mapped reads were generated from each sample.

CUT&RUN and sequencing

Cleavage Under Targets and Release Using Nuclease (CUT&RUN) was performed using the CUT&RUN assay kit (Cell Signaling Technology), following the manufacturer's protocol. Briefly, 100,000 cells were fixed with formaldehyde (0.1%, room temperature, 2 min, with rotation) before binding with the activated Concanavalin A Beads. Antibody against H3K27ac (Active Motif) was added to the cell:bead suspension, followed by the addition of pAG-MNase enzyme, which was subsequently activated by calcium to digest the DNA. The reaction was sequestered using the Stop Buffer that was supplemented with 5 pg of *S. cerevisiae* yeast spike-in DNA and the DNA was extracted and purified. The input DNA was sheared to 100–600 bp fragments using a sonicator (Covaris, Woburn, Massachusetts) before purification. CUT&RUN library was prepared using the SimpleChIP ChIP-seq DNA library Prep Kit for Illumina (Cell Signaling Technology) with Simple ChIP ChIP-seq Multiplex Oligos (Cell Signaling Technology) and sequences on a NextSeq platform for paired-end sequencing with a read length of 75 bp.

ATAC-seq

ATAC-seq was performed as previously described (Corces et al., 2017; Liu et al., 2020). In brief, 50000 cells were seeded in DMEM10 with or without Dox for 5 days, and 50000 cells were pelleted in cold PBS. The cells were lysed by resuspending in 100 µL cold ATAC-resuspension buffer (RSB, 0.1% NP40, 0.1% Tween 20, and 0.01% Digitonin) and incubated on ice for 3 min. The lysis was washed in 1.4 mL of cold ATAC-wash buffer (0.1% Tween 20) and the pelleted nuclei was resuspended in 100 µL of transposition mixture (50 µL 2x TD buffer, 5 µL transposase (100nM final), 33 µL PBS, 1 µL 1% digitonin, 1 µL 10% Tween 20, 5 µL H₂O). After incubation at 37°C for 30 min in a thermomixer (Eppendorf, Enfield, CT) at 1000 rpm, the reaction was cleaned up with a Zymo DNA Clean and Concentrator-5 Kit

(Zymo Research, Irvine, CA) and the DNA was eluted in 21 μ L elution buffer. Nextera DNA Library Prep kit (Illumina) was used for ATAC sequencing library preparation, according to the manufacturer's protocol. The amplified library was right size selected using SPRIselect reagent (Beckman, Brea, CA) following the instruction. ATAC libraries were sequenced on an Illumina NextSeq machine (paired-end 75-bp reads). The Fastq files were processed using Encode ATAC_DNase_pipelines (https://github.com/kundajelab/atac_dnase_pipelines) installed on the NIH biowulf cluster (https://hpc.nih.gov/apps/atac_dnase_pipelines.html).

Antibodies

Antibodies used for western blotting (WB), co-immunoprecipitation (co-IP), chromatin-immunoprecipitation (ChIP) and flow cytometry assays were obtained from different companies with different dilutions used for different assays. Their source and specific dilutions are listed in Table S7.

Transient transfection

Transient transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following a reverse transfection protocol. Briefly, lipofectamine and RNAi duplex were separately diluted in Opti-MEM and then mixed together and incubated at room temperature for 20 min. The mixture was added to the cell culture plate followed by the addition of counted cells. The following small interfering RNAs (siRNA) were purchased from Qiagen (Germantown, MD): AllStars Negative control siRNA (cat. 1027281), siFOXC1-1 (cat. SI00026138), siFOXC1-5 (cat. SI03037713), siHOXB8-4 (cat. SI00440685), siHOXB8-6 (cat. SI04311916).

Customized siRNA library screening

A customized siRNA library was assembled by picking 3 individual siRNA that target different regions of the candidate CRC transcription factor. This was done through the GeneGlobe gene-specific product lookup tool on the Qiagen website (<https://geneglobe.qiagen.com/product-groups/flexiplate-sirna>), and all siRNA products were purchased from Qiagen on a 384-well plate in 0.1 nmol quantities. The stock siRNA (10 μ M) was created by adding 10 μ L of RNase-free H₂O directly into the product and a working solution of 1 μ M was created with further dilution. The siRNA used for the screen was diluted in Opti-MEM to 133.3 nM and 10 μ L of this siRNA dilution was mixed with 10 μ L of lipofectamine RNAiMAX diluted in Opti-MEM directly in a 384-well cell culture plate. A reverse transfection protocol was followed. Cell confluency and morphology were assessed in real time using the Incucyte S3 live cell analysis system (Essen BioScience) for 96 h. Four days after the transfection, cell viability was assessed by using MTS assay with CellTiter 96 AQueous One Solution (Promega, Madison, WI), following the manufacturer's instructions. The median percentage reduction in signal from control siRNA was calculated and visualized in R.

CRISPR-Cas9 mediated gene knockout

To generate MPNST cells that expressed an inducible Cas9 protein, they were transduced with the lentivirus of Edit-R Inducible Lentiviral Cas9 (Dharmacon, Waltham, MA) followed with antibiotic selection by blasticidin (Invitrogen). These cells expressed Cas9 when induced with Dox (0.5 μ g/mL). Two guide RNAs (gRNAs) that target the exon of human *FOXC1* gene or the first exon of human *HOXB8* gene were designed using the online tool offered by Dharmacon. They were synthesized and cloned onto the pLentiGuide-Puro vector (GenScript, Piscataway NJ) to be packaged into lentivirus using the Lenti-X HEK293 cells. The above mentioned Dox-inducible Cas9-expressing MPNST cells were transduced with the lentivirus of these gRNAs and selected with puromycin (Invitrogen). Gene-editing was enabled by Dox treatment and the elimination of target protein was confirmed by western blotting after 5-7 days post treatment.

Soft agar clonogenic assay

To assess effects of *SUZ12* restoration, *FOXC1* knockout and *HOXB8* knockout on anchorage independent cell growth, 1.5×10^5 cells stably transfected with Dox-inducible *SUZ12* expression constructs, Dox-inducible Cas9 and two gRNAs targeting *FOXC1* and *HOXB8* were cultured in 0.7% top agarose in DMEM10 on a layer of 1.4% bottom agarose in DMEM10 to prevent the adhesion of cells to the culture plates. Medium was changed twice a week with or without 0.5 μ g/mL Dox. Colonies with a diameter approximately 200 μ m were counted under a microscope after 4 weeks. Representative colonies were imaged using a Nikon Eclipse TE300 microscope (Nikon, Melville, NY).

Flow cytometry

Dox-inducible *SUZ12*-expressing cell, T265, were seeded then treated with Dox (0.5 μ g/mL) the following day. Two million cells treated with or without Dox were harvested and washed once with flow staining buffer (FSB, PBS supplemented with 2% FBS and 1 mM EDTA) and blocked with anti-Fc receptor (Biolegend) at room temperature for 5 min. One million cells from each condition were stained with PE anti-human HLA-A,B,C or IgG isotype and the fixable viability dye eFluor 506, which were diluted with FSB at room temperature for 12 min. A separate half million cells were incubated on a heat block at 65°C for 1 min to kill the cells, then mixed with the remaining half million cells and stained with the fixable viability dye eFluor 506 alone for compensation. A separate million cells were stained with PE anti-human HLA-A,B,C alone for compensation. Post staining, cells were washed twice with FSB followed by resuspension in 300 μ L of FSB and ready for flow cytometry. The LSRFortessa (BD, Franklin Lakes, NJ) flow cytometer was used to assess the fluorescent intensity of MPNST cells stained for human MHC I molecules. Cells were gated to retain live, single cells for

downstream analysis of the protein of interest in FlowJo software (BD). The stacked intensity of HLA-A,B,C was visualized in FlowJo.

Single-cell RNAseq

Patient MPNST samples were directly collected from the operating room in the Center for Clinical Research of the National Institutes of Health and immediately processed in a laminar flow hood. The tumor was minced into 1 mm³ cubes in tumor dissociation media {DMEM10, collagenase I (STEMCELL Technologies, Vancouver, Canada), dispase II (MilliporeSigma, St. Louis, MO), and DNase I (Invitrogen)}, and transferred to a gentleMACS C Tube (Miltenyl Biotec, Bergisch Gladbach, Germany) and dissociated on a gentleMACS dissociator (Miltenyl Biotec) followed by shaking at 200 rpm at 37°C for 40 min. The dissociated tumor cells were pushed through a 40 μm cell strainer with a syringe plunger, and the cell strainer was rinsed twice with DMEM10. The cells were washed with PBS and counted using the AO/PI viability dye (Nexcelom, Lawrence, MA) in an automated cell counter, Cellometer Auto 2000 (Nexcelom) or LunaFL (Logos Biosystems, Annandale, VA). Ten thousand live cells per capture lane were subsequently captured by the Chromium Controller (10x Genomics, Pleasanton, CA), aiming to capture 6000 live cells per lane, using the v3 reagent for library preparation, following the manufacturer's instructions. Sequencing was done on the Illumina sequencer NextSeq550, aiming for >50000 reads per cell.

QUANTIFICATION AND STATISTICAL ANALYSIS

RNAseq data analysis

Reads of the samples were trimmed for adapters and low-quality bases using Trimmomatic (Bolger et al., 2014) before alignment with the reference genome (Human – hg19) and the annotated transcripts using STAR (Dobin et al., 2013). The average mapping rate of all samples was 97%. Unique alignment was above 86%. There were 1.94–4.20% unmapped reads. The mapping statistics were calculated using Picard software. The samples had 0.83% ribosomal bases. Percent coding bases were between 56 and 61%. Percent UTR bases were 28–34%, and mRNA bases were between 87 and 94% for all the samples. Library complexity was measured in terms of unique fragments in the mapped reads using Picard's MarkDuplicate utility. The samples had 68–78% non-duplicate reads. In addition, the gene expression quantification analysis was performed for all samples using STAR/RSEM (Li and Dewey, 2011) tools. The raw count was used in DESeq2 (Love et al., 2014) for differentially expressed gene analysis. Statistical results of gene expression from DESeq2 were used in gene set enrichment analysis (GSEA) and a false-discovery rate of less than 0.25 was considered a significant enrichment. Gene Ontology (GO) Enrichment analysis was performed using the list of differentially expressed genes against all GO terms. The normalized count, specifically fragments per kilobase of transcript per million mapped reads (FPKM), was used for data visualization in heatmaps. R package ComplexHeatmap (Gu et al., 2016) was used to generate heatmaps in R (version 3.6.1). Raw RNAseq results were aligned to human genome hg19 and converted to bigwig files using deeptools (Ramírez et al., 2016) for visualization in the IGV genome browser (Robinson et al., 2011) together with ChIP-seq data.

ChIP-seq and CUT&RUN data analysis

ChIP and CUT&RUN enriched DNA reads were trimmed for adapters using Trimmomatic software before the alignment. The trimmed reads were aligned to the reference human genome (version hg19) using BWA (Li and Durbin, 2009). Duplicated reads were discarded. Reads aligned to regions of the hg19 genome known to be prone to artifactual increased alignments were removed. For sample visualization in IGV, bam files that contained the alignment reads were carried into deepTools for the generation of coverage track (bigwig files) using the bamCoverage function (Ramírez et al., 2016). Read counts aligned to human (hg19) and Drosophila (dm3) genomes were calculated for the normalization of ChIP-seq spike in. Read counts aligned to human (hg19) and *S. cerevisiae* yeast genomes were calculated for the normalization of CUT&RUN spike in. The coverage was calculated by extending the reads to the average size, which was measured by Agilent Bioanalyzer (Agilent, Santa Clara, CA), and counting the number of reads per bin, where bins were short consecutive counting windows of 25 bp. These bigwig files were also used to plot heatmaps using deepTools and the metagene plot using NGSplot (Shen et al., 2014).

ChIP-seq read density values were normalized per million mapped reads. High-confidence peaks were identified by MACS (Zhang et al., 2008) (version 2.2.6) with the narrow algorithm for FOXC1 and H3K4me3 and broad algorithm for H3K27me3 and H3K27ac. Overlapped H3K27me3 peaks were identified using the H3K27me3 peak files of all three inducible cell lines in the +Dox condition in BEDTools (<https://bedtools.readthedocs.io/en/latest/index.html>) and BEDOPS (Neph et al., 2012). Bivalent domains marked by both H3K27me3 and H3K4me3 are poised for rapid transcriptional control during development (Bernstein et al., 2006). H3K27me3 and H3K4me3 peaks were overlapped at these bivalent sites in the each of the three Dox-induced cell lines. Cell specific bivalent domains were defined if a restored H3K27me3 peak overlapped with H3K4me3 peak within a given MPNST cell line. Intersection of the peaks from the three lines demonstrated 850 common bivalent genomic sites observed in the three cell lines. FOXC1 peaks were segmented into three categories: promoters (within +/- 1000 bp from the transcription starting site), enhancer-bound (overlapped with enhancer bed file in BEDOPS) and others. Peaks were annotated in HOMER (Heinz et al., 2010) with the promoter defined as transcription starting site +/- 1000 bp. Peak-associated genes were used for GO analysis and visualization in R package ReactomePA (Yu and He, 2016). Enrichment of known and *de novo* motifs were first identified using HOMER (Heinz et al., 2010) and confirmed with in-house algorithm.

Enhancers were identified using the Rank Ordering of Super-Enhancer (ROSE) software (Lovén et al., 2013; Whyte et al., 2013) and distal (>2500 bp from transcription starting site) H3K27ac peaks. Enhancer constituents were stitched together if clustered within a distance of 12.5 kb. The enhancers were classified into typical and super enhancers based on a cutoff at the inflection point (where tangent slope = 1) in the rank-ordered H3K27ac ChIP-seq signal, which was normalized to input signal. To identify candidate core transcription factors, CRCmapper was adopted to find autoregulated super enhancer-driven expressed transcription factors (Saint-André et al., 2016). MPNST-specific core transcription factors were defined by comparing published MPNST tissue RNAseq (Lee et al., 2014) and in-house generated MPNST cell line RNAseq data with a panel of normal tissue controls (Chang et al., 2016) to find those significantly highly expressed in MPNSTs ($p < 0.05$ by Student's t-test). The predicted inter-connected transcriptional regulations through MPNST-specific autoregulated super enhancer-driven transcription factors were visualized using the R package circlize (Gu et al., 2014).

Single-cell RNAseq data analysis

The single-cell RNA sequencing results were analyzed preliminarily with the Cell Ranger pipeline offered by the 10X Genomics. The generated count matrix was used in downstream analysis in Seurat 4.0. Briefly, gene counts were normalized and scaled before dimensional reduction for the clustering analysis. Cluster biomarkers were identified by finding the differentially expressed genes in each cluster with comparison to all other clusters. For multiple sample analysis, we used the anchor-based standard integration pipeline in Seurat 4.0 (Butler et al., 2018; Stuart et al., 2019). Generally, subclones that were present in more than one sample were identified and biomarkers that were conserved in the common subclones or unique to each subclone were obtained. Cell types were manually annotated based on the expression of identified marker genes in each cluster. Using the immune cells from these MPNST single-cell datasets as a reference, we inferred copy number variation by using the R package inferCNV (inferCNV of the Trinity CTAT Project. <https://github.com/broadinstitute/inferCNV>).

Single-cell whole genome sequencing and data processing

To profile the genomic dynamic of tumor cells of a patient MPNST, a portion of the dissociated tumor cells from the resected primary MPNST of the first patient that were prepared for single-cell RNAseq were used for single-cell whole genome sequencing. Basically, about 600 live cells per lane were captured in the Chromium controller using the Chromium Chip single-cell CNV chemistry for library preparation (10x Genomics). The library was sequenced on an Illumina NovaSeq6000 platform and a total of 1270 cells were sequenced for an average of 1.8 million reads per cell. The median estimated copy number variation resolution was 0.84 million bp. Sequencing reads were processed and analyzed using the Cell Ranger DNA analysis pipeline (10X Genomics), which included sample demultiplexing, barcode detection, read alignment, copy number estimation on single-cells and groups of cells defined by hierarchy clustering. The hierarchically clustered single-cell copy number profiling and genomic variability across the genome were visualized in Loupe scDNA Browser (10X Genomics). To definitively determine the malignant cell populations in the tumor, results from the single-cell whole genome sequencing were subsequently compared to results from the inferred copy number profiling generated from the using single-cell RNAseq results.

Construction of a normal schwann cell developmental trajectory

Data download and processing

Read count matrices of E9.5 neural crest cells {GSE129114 (Soldatov et al., 2019)}, E12.5/E13.5 adrenal medulla cells {GSE150150 (Furlan et al., 2017)}, and mature Schwann cells {GSE142541 (Wolbert et al., 2020)} were downloaded from the GEO database. Published cell-type annotations were used for GSE129114 (Table S9 in source publication) and GSE142451. For GSE150150, batch correction and clustering were performed using the default parameters in Seurat, and annotated cell types were identified based on marker genes employed in the source publication.

After all datasets were merged together, marker genes were derived for each cell type using the FindMarkers function in Seurat using the built-in logistic regression-based algorithm. The library size (sequencing depth in each cell), cell cycle status, and batch were used as co-variables to estimate the p value of differential expression for each gene. Genes with an adjusted p value of less than 0.1 were retained as signature genes for each cell type, with at most 300 genes in each signature. The activity of each of the signature gene set was scored using AUCell, which is a part of the SCENIC package (Aibar et al., 2017). The AUCell activities were compared between malignant MPNST cells and normal Schwann cells from the patient MPNST single-cell dataset using two-sided Wilcoxon test.

Trajectory analysis

Since data from GSE129114 and GSE150150 represented a much greater library size than GSE142541, reads were down-sampled from both these datasets to match the library size distribution of cells in GSE142541. Monocle3 (Cao et al., 2019; Trapnell et al., 2014) was then used to construct a trajectory from the merged data, where batch correction was internally performed before pseudotime values were computed for each cell. E9.5 neural tube cells were chosen to represent the root state of the trajectory and were assigned a pseudotime value of zero.

Regulon analysis

SCENIC (Aibar et al., 2017) was used to derive regulons, i.e., a target gene set for each TF, active in each developing cell type, based on the presence of binding sites within 10 kb of the TSS of expressed genes. TFs whose mean regulon activity was at least 0.03 in at least one of the cell types were retained for further analyses.

Motif analysis in promoter and enhancers

Promoters and enhancer regions were scanned with a custom script that relied on the SPRY-SARUS motif scanner (Kulakovskiy et al., 2016). A total of 816 motifs from a merged collection of HOCOMOCO v11 and JASPAR 2020 databases were used (Fomes et al., 2020; Kulakovskiy et al., 2016). During the merging process, if the motif for the same TF was present in both collections, the HOCOMOCO motifs were retained since these are known to correlate highly with ChIP-seq peaks (Kulakovskiy et al., 2016). During the motif scan process, those TFs that had a non-zero average expression were chosen within the MPNST and Proliferating clusters.

Those genomic regions spanning 1 kb upstream and 200 bp downstream of transcription start sites were chosen as promoters. The stitched H3K27ac peaks that were the output of the ROSE algorithm were used as enhancers. Motif enrichment in promoters and enhancers was separately computed but the same methodology was used for both classes of regions.

Out of the motifs in our collection, those 651 motifs whose TFs had a mean expression above zero in the MPNST and Proliferating clusters were used. For an input set of foreground genomic regions, a dinucleotide-shuffled set of background regions was generated using the Euler algorithm from TFBSTools (Tan and Lenhard, 2016). For each motif, the total number of motif matches in the foreground and background using a motif score threshold that corresponded to a p value of 10^{-4} was calculated. Then the fold-enrichment, F , for the motif was defined as the ratio between the number of binding sites in the foreground set to that of the background set. Multiple binding sites were allowed to occur in each sequence. Since the lengths of genomic regions in the foreground and background set are identically distributed, the value of F controls for the fact that super-enhancers, owing to their larger size than typical enhancers and promoters, are more likely to contain false positive binding site matches.

Statistical analysis

The statistical methods used in this study were specified in the corresponding Results and Methods sections. Statistical analysis was performed in R, Microsoft Excel, and GraphPad Prism software (GraphPad, San Diego, CA).