2025 CCF Symposium Poster Session – 1:00 p.m. – 2:45 p.m.

Please note: Session A posters will ONLY be viewable during Session A and
Session B posters will ONLY be viewable during Session B, as they will replace the Session A posters.

Session A Posters: 1:00 p.m. - 1:45 p.m.

No.	Last Name	Institution	Title
1	Allegakoen	Georgetown	EWS::FLI1 transcriptionally represses RBPMS to promote oncogenic transformation in Ewing sarcoma
2	Ayanlaja	Hopkins	Combined MEK Inhibition and RAF Dimer Disruption Overcomes Feedback-mediated Reactivation in ERK-Addicted High-Grade Gliomas
3	Baker	Hopkins	Sensitivity to SHP2 inhibition is dependent on RAS mutation status and is augmented through the addition of combined pharmacologic RAS-GTP inhibition.
4	**Biermann***	**Georgetown**	**Growth-associated protein 43 (GAP-43) upregulation in hypoxic Ewing sarcoma cells and its link to bone metastasis.**
5	Cantilena	NCI	The Impact of Prior Immunotherapy on Composition of Apheresis Material for CAR T-cell Therapy in Patients with B-Cell Acute Lymphocytic Leukemia (B-ALL)
6	**Choe**	**Hopkins**	**Anti-B7-H3 chimeric antigen receptor NK cells show antigen specific cytotoxicity against atypical teratoid / rhabdoid tumors in vitro and in vivo**
7	Culbert	NCI	Infectious Complications After CAR T-cell Therapy for Pediatric and Young Adult Acute Lymphoblastic Leukemia: a single-center experience
8	**Dangi**	**Hopkins**	**Investigating the abnormal activation of the integrated stress response in atypical teratoid/rhabdoid tumor cell lines treated with the PI3K inhibitor paxalisib and the nucleoside analog gemcitabine**
9	Deniz	Georgetown	A novel function of “inactive” cytoplasmic ezrin in its closed conformation as an RNA binding protein
10	**Dey**	**NCI**	**A Second-Generation XPO1 Inhibitor, Eltanexor, Demonstrates Potent Activity Against PAX3::FOXO1 Fusion Positive Rhabdomyosarcoma**
11	**Di Giulio**	**NCI**	**A Tale of States: Decoding the Role of MYCN Amplification in ADRN-MES Plasticity**
12	**Dionysiou**	**Hopkins**	**miR-21 Regulates T-Cell Alloreactivity and GVHD Severity in Experimental Allo-HCT**
13	Dreyzin	NCI	Early leukapheresis in patients with B-ALL yields an activated, early memory T-cell phenotype associated with response to CAR T-cell therapy
14	Dufek	NCI	Successful Implementation of Mock Magnetic Resonance Imaging (MRI) in Children with Neurofibromatosis Type 1 Requiring General Anesthesia for MRIs
15	Fang	NCI	KAT2A and MYCN interact to drive oncogenic transcriptional regulation in neuroblastoma
16	Ferguson	NCI	Angubindin-1-Mediated Disruption of Brain Endothelium Enhances T-cell Recruitment and Drug Delivery in Rodent Glioma Models
17	Flamourakis	Hopkins	Metabolic profiling of MEKi-treated fusion-negative rhabdomyosarcoma cell lines suggests a role for glutamine antagonism as a preclinical therapeutic approach.
18	**Groff**	**Hopkins**	**Developmental states determine intrinsic resistance to immunotherapy in pediatric rhabdomyosarcoma**
19	Haffey	Children's National	Dual-Targeting TCR-CAR T Cells for Enhanced Immunotherapy in High-Grade Gliomas
20	Hoang	NCI	The in vivo dynamics of CD22 CAR T-cells: pharmacokinetic approach to cellular therapy monitoring

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2025 CCF Symposium Poster Session – 1:00 p.m. – 2:45 p.m.

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21	Hogan	NCI	Emerging Themes in the Perinatal and Early Medical History in a Cohort of Patients with MEN2B Followed at the National Institutes of Health
22	**Holl**	**Hopkins**	**Chimeric cytokine receptors increase NK-cell functionality against AML**
23	Hutchison*	Hopkins	Cognitive evaluation following image-guided whole brain proton FLASH
24	Johnson	Hopkins	Characterizing and Isolating a Senescent Stem-like Blast Subpopulation in Philadelphia Chromosome-like Acute Lymphoblastic Leukemia
25	**Kang**	**NCI**	**Stag2-cohesin mediates 3d genome dynamics to regulate oncogenic transcription in neuroblastoma**
26	Kaschak	NCI	Longitudinal Follow-Up and Outcomes of Pediatric and Adult Patients with SDH-deficient GIST
27	Kim	NCI	Endogenous HiBiT-tagging of PAX3::FOXO1 reveals that CDK inhibitors downregulate the fusion oncogene and disrupt core regulatory transcription in PAX3::FOXO1 positive rhabdomyosarcoma
28	Knoll	Georgetown	SETD2 Mediates EWS::FLI1 Tolerance in Non-Ewing Sarcoma Cells
29	Lee	Hopkins	BCL-6 corepressor-DNMT1 complex modulates IGF-1 transcription in retinoblastoma
30	Liang	Hopkins	Combining MEK and CDK4/6 Inhibition: Synergistic Treatment of ATRT with Mirdametinib and Abemaciclib
31	**Liu**	**NCI**	**Lineage-Specific Transcription Factor Screen Identifies TFs That Regulate Neuroblastoma Plasticity and Drug Resistance**
32	Liu	NCI	Deciphering the code of MYCN protein in neuroblastoma: unveiling its secrets through barcoded mutagenesis screen and single-cell RNA sequencing analysis
33	Mahmoud	NCI	Metastatic PDX models of Ewing Sarcoma: Development and Characterization

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Session B Posters: 2:00 p.m. – 2:45 p.m.

No.	Last Name	Institution	Title
34	**Khorsandi**	**Children's National**	**Dual CAR T-Cell Therapy Targeting B7-H3 and CD123 Remodels the Tumor Microenvironment in Pediatric Glioma**
35	Lemberg	Hopkins	Acquired resistance to MEK inhibition alters glutamine metabolism and promotes therapeutic vulnerability to the glutamine amidotransferase inhibitor DRP-104 in NF1-associated malignant peripheral nerve sheath tumor
36	Makri	Hopkins	Advancing NF1 research through a comprehensive biorepository of primary tumor specimens, preclinical models, genomic and clinical data for NF1-associated tumors
37	Zhang	Hopkins	Combined SHP2 and CDK4/6 inhibition depletes intratumoral tumor-associated macrophages in malignant peripheral nerve sheath tumors
38	McGuire	Georgetown	Evaluating the role of splicing in Ewing Sarcoma using an SRRM2-GFP knock-in experimental system
39	Milewski*	NCI	Engineered T cell therapy in combination with TGF-beta signaling blockade for immunotherapy against osteosarcoma
40	Mills	NCI	Multiplex Staining and VINE-seq Demonstrates the Temporospatial Complexity of the Blood-Tumor-Barrier in Pediatric-Type Diffuse High Grade Glioma
41	Molotkova*	Georgetown	ROME, a novel membrane protein that accelerates Ewing sarcoma metastasis
42	Mulvaney*	VT/CNRI	CRISPR-Drug Combinatorial Screening Identifies Effective Combination Treatments for MTAP null cancer
43	Odeniyide	Hopkins	Pan-RAS inhibition as a novel therapeutic strategy in RAS-driven rhabdomyosarcoma
44	**O'Koren**	**NCI**	**Disparities between bone marrow B-cell aplasia and minimal residual disease in pediatric and AYA B-ALL after CAR T-cell therapy**
45	**Reyes-Gonzalez**	**NCI**	**Genome-wide CRISPR-Cas9 screening to interrogate mechanisms of CAR T-cell resistance in neuroblastoma**
46	Sachs	NCI	Secretome distinguishes spectrum of NF1 associated peripheral nerve sheath tumor
47	Sayers	NCI	FHD-286, a small molecule inhibitor of the SWI/SNF ATPases SMARCA2/4 blocks neuroblastoma phenotypic switching
48	Selvanathan	Georgetown	A novel human mesenchymal stem cell model of Ewing sarcoma recapitulates EWS::FLI1 associated protein interactome involved in mRNA splicing and chromatin remodeling BAF complex
49	Shah	Georgetown	Molecular Mechanisms of PROTAC-Induced Target Degradation in Fusion-Positive Rhabdomyosarcoma
50	Shaw	Georgetown	Determinants of chemotherapeutic resistance in fusion-positive rhabdomyosarcoma
51	**Siegel**	**Children's National**	**Long-term hematologic effects of selumetinib treatment in children with inoperable plexiform neurofibromas**
52	Singh	Hopkins	The Methylcytosine Dioxygenase Tet2 influences outcomes of Graft-versus-Host Disease through regulation of Donor T-cells
53	Sundby	NCI	Deep Learning Predicts CDKN2A/B status from H&E-Stained Whole Slide Images in Peripheral Nerve Sheath Tumors

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54	**Tardito**	**Children's National**	**Immunosuppressive 3D tumor models: pioneering combinatorial T cell therapies.**
55	Tindall	Hopkins	Novel Agent LP-184 and Spironolactone Extend Mouse Survival in Atypical Teratoid Rhabdoid Tumor
56	Tiwari	Georgetown	Characterizing RNA Binding of Open and Closed Ezrin
57	Tremble	NCI	Transcriptional regulation of Neuroblastoma cellular heterogeneity by STAG1- and STAG2-cohesin
58	Tufiño	Georgetown	Elucidating the Interaction of Small-Molecule Inhibitor (S)-YK-4-279 and EWS::FLI1
59	Vaidyanathan	Hopkins	Dissecting the molecular mechanisms driving Lineage plasticity in High-Risk Pediatric ALL
60	VanNoy	Children's National	Targeting the CAR T Cell DNA Methylome to Enhance Persistence and Efficacy in Pediatric Brain Tumors
61	Wang	Hopkins	Mirdametinib and Paxalisib Suppress Pediatric Low-Grade Glioma Growth
62	Wang	Children's National	Cell-free DNA shallow-pass whole genome sequencing as a non-invasive biologic assay to track non-CNS extramedullary B-ALL
63	Wei	NCI	Potent FGFR4-targeted antibody-drug conjugate (ADC) therapies for patients with rhabdomyosarcoma and other human cancers
64	**Yaacoub**	**Children's National**	**Reprogramming Tumor-Associated Macrophages to Enhance CAR T Cell Therapy in Group 3 Medulloblastoma**
65	**Yu**	**NCI**	**Evaluating in vivo efficacy of NAMPT inhibitor OT-82 in preclinical neuroblastoma models**
66	Zhang	Hopkins	The CoREST inhibitor, corin, suppresses proliferation, increases apoptosis and promotes differentiation in malignant rhabdoid tumors
67	Zinsky	Hopkins	Defining Phenotypic and Functional Differences of Activated NK Cells to Improve Cancer Immunotherapy

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Please Note: The following text was compiled from numerous PDF forms, so formatting and presentation style will vary slightly. The Children's Cancer Foundation, Inc. has made no substantive edits, only minor formatting ones.

SESSION A POSTERS

1

EWS::FLI1 transcriptionally represses RBPMS to promote oncogenic transformation in Ewing sarcoma

David Allegakoen, MS (Georgetown University) Saravana Selvanathan, PhD (Georgetown University) Jeffrey Toretsky, MD (Georgetown University)

Background: Ewing sarcoma is a pediatric bone and soft tissue cancer thought to arise from mesenchymal stem cells (MSCs). It is characterized by fusion of the RNA-binding protein EWS to an ETS transcription factor, most often FLI1. Recent work has shown that EWS::FLI1, in addition to altering gene expression, induces changes in mRNA splicing and 3D genome organization that may also contribute to transformation and proliferation.

By affinity purification mass spectrometry, our lab showed that EWS::FLI1 could interact with proteins involved in chromatin remodeling, transcription, and splicing. Subsequent proximity biotinylation studies by another group revealed that EWS::FLI1 engaged three major complexes in Ewing sarcoma cells: the chromatin remodeling BAF complex, the transcriptional Mediator complex, and the spliceosome, suggesting a direct role in all three functions.

In MSCs, however, our lab found that EWS::FLI1 maintained interactions with fewer BAF complex and chromatin-associated proteins and had additional interactions with RNA-binding proteins and ribosomal components. Analysis of the transcriptional state of these MSCs showed limited similarity to the gene expression and splicing signatures observed in Ewing cell lines.

Results: To identify RNA-binding proteins that might limit EWS::FLI1 activity in MSCs, I performed differential expression analysis between Ewing and MSC lines. I found that RBPMS, which was previously suggested to interact with EWS::FLI1, was among the most strongly downregulated RNA-binding proteins and that its downregulation was EWS::FLI1 dependent.

In Ewing cell lines, I found that RBPMS overexpression modestly shifted gene expression and splicing profiles to a more MSC-like state; however, there was limited effect on adherent or soft agar growth. In MSCs, I found that knockdown of RBPMS enabled higher

EWS::FLI1 expression, increased soft agar growth, and a slight shift towards more Ewing-like gene expression and splicing.

Conclusions: Together, these data suggest that RBPMS might act as a barrier to establishment but not maintenance of EWS::FLI1-dependent transformation. RBPMS knockdown appears to allow increased repression of EWS:: FLI1-repressed targets and increased induction of TP63 and its downstream targets. Future work will focus on determining the mechanism and impact of these effects.

2

Combined MEK Inhibition and RAF Dimer Disruption Overcomes Feedback-mediated Reactivation in ERK-Addicted High-Grade Gliomas

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Background: High-grade gliomas (HGGs) are aggressive, incurable malignancies with frequent alterations in PI3K or ERK signaling pathways, leading to potential targets for therapeutic exploitation. While MAPK/ERK pathway alterations drive oncogenic ERK signaling in some HGGs, single-agent MEK inhibitors (MEKi) yield transient responses at best due to adaptive resistance. Mechanistically, MEKi disrupts negative feedback loops mediated by RAS-GAPs and DUSPs, triggering compensatory RAF dimerization and ERK reactivation. To address this, we hypothesized combining the MEKi mirdametinib with the RAF dimer-disrupting inhibitor (RAFi) BGB-3245 would improve vertical ERK pathway inhibition. This study evaluates the preclinical efficacy of this combination in ERK pathway-addicted HGGs.

Methods: Given the limited pediatric HGG models, adult GBM neurosphere and adherent cell lines with diverse genetic backgrounds (e.g., EGFR amplification, RB/PTEN loss, NF1/TP53 loss) were used, due to shared oncogenic dependencies, particularly in MAPK/ERK-driven tumors. Cells were treated with mirdametinib, BGB-3245, or their combination. RNA sequencing (RNAseq) identified differential gene expression and

pathway changes post-MEKi treatment. Growth inhibition, pathway activity, and apoptosis were quantified through live-cell imaging and immunoblot analysis. Synergy was evaluated using Loewe's method. Heterotopic patient-derived xenografts and orthotopic cell-line models were utilized to determine the in vivo efficacy, measuring tumor growth and ERK pathway inhibition.

Results: RNAseq analysis revealed significant downregulation of canonical RAS-MAPK signaling and diminisshed regulation by RAS-GAPs and downstream DUSPS post-MEKi, consistent with loss of feedback inhibition.

Concurrently, MEKi triggered RTK pathway reprogramming, further promoting RAS activation. Functional studies revealed that combining mirdametinib and BGB-3245 results in superior tumor-suppressive effects compared to monotherapy across multiple HGG lines. The combination enhanced ERK pathway inhibition and apoptosis induction, with synergy observed across all models, including NF1-deficient lines. In vivo, combination therapy reduced tumor growth and ERK pathway activity in heterotopic but not in orthotopic xenografts.

Conclusion: The combination of MEKi with a RAF dimer-disrupter has therapeutic potential for gliomas with a variety of genetic backgrounds given the common reliance on ERK signaling. Dual inhibition prevents loss of feedback inhibition and deepens ERK suppression, but drugs with adequate brain penetration are needed for further evaluation in HGG.

3

Sensitivity to SHP2 inhibition is dependent on RAS mutation status and is augmented through the addition of combined pharmacologic RAS-GTP inhibition.

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2. Laboratory of Cell and Developmental Signaling, National Cancer Institute, National Institute of Health, Frederick, Maryland.

3. Division of Pediatric Hematology and Oncology, Oregon Health and Science University, Portland, OR

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma. A subset of RMS lacking the PAX3/7:FOXO1 fusion, known as fusion-negative RMS (FN-RMS), is driven by oncogenic point mutations in genes that regulate the activity of the RAS/ERK signaling pathway. Oncogenic mutations in HRAS, NRAS, and KRAS, commonly at codons 12, 13, and 61, slow GTP hydrolysis and prevent effective regulation via GAPs and GEFs. Molecular strategies to target RAS activation in cancer include inhibitors of RAS regulatory proteins, including the non-receptor protein tyrosine phosphatase SHP2. SHP2 promotes RAS signaling by recruiting the GEF SOS1 and by dephosphorylating RAS tyrosine residue 64 (Y64), increasing RAS-GTP levels and promoting ERK signaling. We set out to determine whether SHP2 inhibitors (SHP2i) demonstrate preclinical activity in models of RAS-driven FN-RMS. We hypothesize that mutations in H/N/KRAS, affecting codons 12, 13 and 61, slow GTP hydrolysis and reduce the need for SHP2-mediated RAS re-activation, thus determining SHP2 inhibitor sensitivity. We tested the effects of small molecule SHP2i on ERK signaling in a panel of FN-RMS cell lines with varied RAS mutations. In cells with RTK-amplification (WT RAS), or G12 or G13-mutant HRAS, but not in those with Q61-mutant HRAS or NRAS, SHP2i caused reductions in ERK signaling and in 3-D cell growth. In vivo, SHP2i was active against subcutaneously implanted RAS WT cell line-derived xenografts, but not Q61-mutant xenografts. To confirm RAS mutation status determines SHP2i sensitivity, we created a RAS-mutant isogenic system in murine C2C12 myoblasts and tested its sensitivity to SHP2i. In C2C12 cells expressing WT, G12X, and some G13X, but not Q61X H/N/KRAS, SHP2i caused significant growth inhibition and decreases in p-ERK. In order to improve response to SHP2i, we hypothesized that the combination of SHP2i with a compound that directly inhibits RAS-GTP could be additive. The RAS-GTP inhibitor RMC-7977 inhibits RAS by binding Cyclophilin A (CYPA) to RAS via RAS Y64. We therefore hypothesize that increased RAS Y64 phosphorylation following SHP2i would increase RMC7977-mediated RAS-CYPA interaction, improving effective RAS inhibition. Using the C2C12 isogenic cell lines and treating with RMC-7977 and SHP2i, we found that the combination led to further reductions in p-ERK signaling and cell viability in the majority of H/N/KRAS-mutant cell lines, including Q61-mutant lines, than either compound alone. Work is ongoing to determine the efficacy of the combination in pre-clinical models of FN-RMS.

4

Growth-associated protein 43 (GAP-43) upregulation in hypoxic Ewing sarcoma cells and its link to bone metastasis.

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Ewing Sarcoma (ES) is a pediatric tumor that arises in bone or soft tissues. ES bone metastases do not respond well to routine therapies and their frequency markedly increases upon disease recurrence, which results in a lower survival rate in patients with osseous metastases as compared to those with pulmonary dissemination. Previously, we have shown that ES bone metastasis is initiated by tumor hypoxia, which leads to the formation of polyploid ES cells, the progeny of which, named HYP-4n cells, exhibit high levels of chromosomal instability, metastasize almost exclusively to bone and are less sensitive to cytostatic agents. In this study, we demonstrate that HYP-4n cells exhibit an upregulation in several neuronal proteins, including growth-associated protein 43 (GAP-43), a membrane protein promoting axon growth in neurons and formation of microtubes connecting glioblastoma cells into invasive and therapy-resistant network. Similar phenotype characterized by the presence of microtube-like cell-cell connections was observed in ES HYP-4n cells. GAP-43 immunostaining in HYP-4n cells revealed a punctuated pattern along the protrusions that is similar to its localization in neurons. Western blot analysis performed on HYP-4n cells revealed a membranous localization of GAP-43, which was not detectable in their normoxic diploid counterparts. The functional role of the GAP-43-positive protrusions in connecting HYP-4n cells was confirmed by the gap-junction permeable dye transfer between the cells and ion transfer detected by a patch clamp assay. DAPI and mitotracker staining detected nuclear fragments and mitochondria within the protrusions, suggesting their transfer between the cells. The suppression of GAP-43 expression via siRNA inhibited HYP-4n cell migration measured via transwell assay. In immunostained ES xenografts, GAP-43 expression was shown in HYP-4n primary and osseous metastatic tumors, with the highest membranous expression in the ES cells on the invading edge of the tumors. A similar pattern of expression of GAP-43 was noted in the human samples of ES bone metastases. This study indicates that GAP-43 is active in the membrane in HYP-4n cells and drives the formation of microtube networks promoting cell invasion. Further studies are required to prove directly the role of GAP-43 in ES bone metastasis and chemoresistance.

5

The Impact of Prior Immunotherapy on Composition of Apheresis Material for CAR T-cell Therapy in Patients with B-Cell Acute Lymphocytic Leukemia (B-ALL)

Anna Cantilena (NIH CC CCE), Bonnie Yates (NIH NCI), Ping Jin (NIH CC CCE), Nirali Shah (NIH NCI), David Stroncek (NIH CC CCE), Alexandra Dreyzin (NIH CC CCE)

Background: CAR T-cell functionality relies on the composition of the apheresis product used for manufacturing, with balanced CD4/CD8 ratios associated with improved patient response. The impact of prior immunotherapy, like blinatumomab and inotuzumab, on apheresis composition has not been established. As these drugs are increasingly used for leukemia therapy, an understanding of how these agents affect apheresis composition will become increasingly important for treatment planning.

Aim: To describe the relationship between blinatumomab and inotuzumab exposure and apheresis composition in patients with r/r B-ALL.

Methods:Data from children and young adults with r/r B-ALL who underwent apheresis collection for CAR T-cell therapy trials (NCT05442515, NCT03448393, NCT02315612) were analyzed retrospectively. Independent variables included exposure to blinatumomab and/or inotuzumab therapies and timing between immunotherapy and apheresis. Any immunotherapy that was given prior to HSCT was excluded. Patients who received both immunotherapies were grouped based on the most proximal therapy. Primary outcomes were total nucleated cell (TNC) yield per patient weight in kilograms, CD3%, and CD4/CD8 ratios measured by flow cytometry. Outcomes were compared using Mann Whitney U tests.

Results: A total of 114 patients with a median age of 18 years (range 4-38), 5 prior lines of therapy (range 2-13), and baseline disease burden of 1% blasts (range 0 to 97%) in bone marrow were evaluated. 29 (25%) patients had blinatumomab exposure at a median of 5.1 months (range 1-58). 22 (19%) had prior inotuzumab at a median of 3.4 months (range 1-30 mo) prior to apheresis .

Exposure and timing of blinatumomab were not associated with differences in apheresis composition. In contrast, inotuzumab exposure at any time prior to apheresis was associated with a lower CD4/CD8 ratio (0.77 for those exposed vs 1.11 for those not exposed, $p = 0.0047$). The timing of inotuzumab did not impact apheresis composition. TNC yield and CD3% did not differ with blinatumomab or inotuzumab exposure.

Conclusion: Inotuzumab exposure may alter CD4 and CD8 proportions in apheresis products, leading to decreased CD4/CD8 ratios, while blinatumomab exposure does not

have the same effect . More detailed characterization of T-cell phenotypes and fitness after immunotherapy will help guide apheresis timing.

6

Anti-B7-H3 chimeric antigen receptor NK cells show antigen specific cytotoxicity against atypical teratoid / rhabdoid tumors in vitro and in vivo

Jun Choe, Sachiv Chakravarti, Natalie Holl, Ruyan Rahnama, Megan Zinsky, Danielle G. Jones, Stamatia Vorri, Adrianna Amaral De Aragao Mendes, Shramana Guchhait, Calixto-Hope Lucas Jr., Eric H. Raabe, Challice L. Bonifant

Johns Hopkins School of Medicine

Background: Atypical teratoid/rhabdoid tumors (AT/RTs) are the most common malignant CNS tumor in infants. AT/RT patients have a 5-year overall survival rate of ~35% and high rates of relapse, emphasizing a dire need for new safe and effective therapies. These therapy-resistant tumors frequently overexpress cell surface molecule B7-H3 (CD276). CAR-NK cells have several advantages over CAR-T cells. NK cells can be obtained from healthy donors and produced as an “off-the-shelf” product. NK cells also have a lower risk of inflammatory toxicity and graft-versus-host disease compared to T cells when transferred across the HLA barrier from healthy donors to patients.

Methods: We have designed a library of variable affinity B7-H3-targeted CARs, produced replication incompetent γ-retroviral vector, and used this for generation of B7-H3 CAR-NK cells. We verified B7-H3 expression in a panel of AT/RT cell lines, and further engineered firefly luciferase expressing AT/RT (CHLA06.ffLuc, BT12.ffLuc, BT37.ffLuc) as well as a CHLA06-derived B7-H3 knockout. We tested CAR-NK cell functionality using in vitro co-culture cytotoxicity assays and developed orthotopic CHLA06.ffLuc and BT12.ffLuc xenograft models for in vivo study. NK cell treatments were delivered intratumorally and monitored for tumor growth and animal survival.

Results: B7-H3 targeted CAR-NK cells demonstrate target-specific cytotoxicity when compared to untransduced (UTD) NK cells. CRISPR/Cas9 mediated knockout of B7-H3 in target cells abolished the difference in CAR vs. no-CAR NK-mediated target killing. When delivered intracranially to CHLA-06 orthotopic xenograft bearing mice, B7-H3 CAR NK cells eliminated tumor cells and prolonged survival. However, antitumor effect of CAR-NK cells was less evident in BT12 xenograft bearing mice. Examination of brains and spines by histology revealed a significant proportion of PBS and UTD NK treated mice with spinal metastases of tumor. When survival data was stratified by presence or absence of spinal metastases, we observed a significant survival advantage for mice without metastases.

Conclusions: Targeting AT/RTs with an anti-B7-H3 CAR-NK cell therapy may provide a safe and effective treatment for patients who have extremely limited therapeutic options. Further study is needed to determine factors that contribute to NK cell efficacy in brain tumors.

7

Infectious Complications After CAR T-cell Therapy for Pediatric and Young Adult Acute Lymphoblastic Leukemia: a single-center experience

August Culbert,*1 Flavia Mesquita-Gava,* 1 Joseph Rocco, 2 Veronique Nussenblatt,2 Grace Li, 1 Brynn B. Duncan, 1 Sara K. Silbert, 1 Haneen Shalabi, 1 Bonnie Yates, 1 Nirali N. Shah1

(*co-contribution)

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Background: Despite the curative potential of chimeric antigen receptor (CAR) T-cells in B-cell acute lymphoblastic leukemia (B-ALL), efficacy can be limited by life-threatening toxicities such as severe infections. Particularly relevant to B-ALL, immune effector cell-associated hematotoxicity-(ICAHT) may be profound, predisposing patients to further risk of infection. This relationship has not been fully assessed in B-ALL patients post-CAR T-cells.

Objective: To profile infectious complications in pediatric and young adult B-ALL patients treated with CAR T-cell products at the National Cancer Institute (NCI) and evaluate patient-specific factors, including ICAHT, for associations with infection risk.

Methods: This retrospective analysis captured data through day+60 (D+60) post-infusion in patients treated with anti-CD19, CD22 CAR-T-cells from 2012-2024. Infections were identified by microbiology, histopathology, or as a clinical syndrome; categorized as bacterial, viral, or fungal; and graded per CTCAEv5.0. Severe infections were defined as grade ≥3. Statistical significance was determined by $p<0.05$ based on two-sided Wilcoxon-rank sum, Pearson's Chi-squared, or Log-rank tests.

Results: Across 160 patients, 49 (30.6%) patients developed 71 infections within 60 days. Viral infections were most common (31, 43.7%), followed by bacterial (29, 40.8%). Severe infection occurred in 23 patients (14.4%), with one fatal bacteremia. Of these infections, 23

(32.4%) were severe; 20 (87%) were bacterial. Patients with infections had higher baseline bone marrow (BM) disease burden (59% vs. 16% blasts) and were more likely to develop prolonged neutropenia with severe ICAHT (grade ≥3) (67% vs. 46%). In multivariable analysis, pre-infusion factors associated with severe infection included older age (OR 1.08 [1.01 – 1.16]), prior infection within 100 days of lymphodepleting chemo (OR 3.23 [1.13 – 10.6]), and higher BM disease burden (OR 1.02 [1.00 – 1.04]). When stratified by BM disease status, ≥5% blasts predicted severe infection (HR 3.2 [1.4 – 7.3]).

Conclusion: 30.6% of children and adults developed infection within 60-days after CAR-T-cell infusion. Most infections were viral, and rates of severe infection were low. In multivariable analysis, high BM disease burden, prior infection, and older age were associated with severe infections. Although post-infusion ICAHT corresponded with severe infections, the discordance between high ICAHT (52.5%) and the low proportion of severe bacterial infections warrants further investigation.

8

Investigating the abnormal activation of the integrated stress response in atypical teratoid/rhabdoid tumor cell lines treated with the PI3K inhibitor paxalisib and the nucleoside analog gemcitabine

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Atypical teratoid/rhabdoid tumors (AT/RT) are highly aggressive central nervous system (CNS) malignancies primarily affecting young children, with a poor four-year event-free survival rate of 37%. These tumors are characterized by aberrant mTORC1/2 pathway activation. Paxalisib, a PI3K inhibitor, efficiently crosses the blood-brain barrier, targeting both mTOR complexes and significantly prolonging survival in AT/RT xenograft models (CHLA-06: 22 to 82.5 days, $p<0.0001$; BT-37: 56 to >100 days, $p<0.0001$). Gemcitabine, a nucleoside analog, inhibits DNA synthesis and activates the integrated stress response (ISR), a cellular pathway regulating gene expression under stress. Elevated eIF2α protein expression and ATF4 mRNA levels in AT/RT suggest ISR dependence for survival. We hypothesize that Paxalisib and Gemcitabine combination treatment disrupts ISR homeostasis, favoring cytotoxicity and tumor cell death.

AT/RT cell lines (CHLA-05, CHLA-06, BT-37) were treated with Paxalisib (450 nM for CHLA-05/CHLA-06, 600 nM for BT-37), Gemcitabine (5 nM), or both for 4 and 24 hours. Apoptosis was assessed in CHLA-06 and BT-37 at 24 and 48 hours via cleaved PARP (cPARP) detection and the MUSE Annexin V assay. Western blot analysis showed increased phospho-eIF2α and reduced ATF4 levels at 4 hours in all three cell lines, with suppression

persisting at 24 hours despite significant apoptosis in CHLA-06 and BT-37. Paxalisib inhibited mTORC1/2, reducing phospho-S6 and phospho-Akt, likely contributing to ATF4 suppression. Gemcitabine-induced ER stress and/or DNA damage activated eIF2α phosphorylation. Yet, the downstream ATF4 remained suppressed, suggesting ISR disruption impaired the tumor cells' cytoprotective response, increasing apoptosis under combination treatment.

Gemcitabine-induced ER stress and/or DNA damage activated eIF2α phosphorylation, which would normally promote ATF4. Paxalisib blunted gemcitabine-induced ATF4 induction. ATF4 suppression despite increased phospho-eIF2α suggests ISR disruption, impairing the tumor cells' ability to mount a cytoprotective response, thereby enhancing apoptotic susceptibility under combination treatment. These findings reveal a novel mechanism by which paxalisib and gemcitabine synergistically disrupt ISR dynamics, suppressing ATF4 and pushing tumor cells toward apoptosis. The resulting loss of ISR homeostasis suggests this combination may enhance AT/RT tumor cell sensitivity to treatment. These results support the potential inclusion of this therapy in upcoming clinical trials for relapsed/recurrent AT/RT.

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A novel function of “inactive” cytoplasmic ezrin in its closed conformation as an RNA binding protein

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Ezrin is a cytoplasmic protein that can exist in multiple conformational forms, which are regulated by a posttranslational modification. Phosphorylation at the threonine 567 residue confers ezrin an open form by abrogating the head-to-tail interaction. Open ezrin was generally accepted as the active form because it can translocate to the plasma membrane and link actin cytoskeleton to the membrane proteins. Closed ezrin, sequestered in the cytoplasm was considered inactive. Our earlier work established that ezrin directly interacts with RNA binding proteins (RBPs). Here, we show that ezrin expression correlates with cytoplasmic RBPs in human osteosarcoma samples. We therefore, hypothesized that closed ezrin is not a dormant protein, but rather biologically active. Purified recombinant ezrin protein in its closed conformation directly bound RNA with 6.1 nM affinity. Ezrin binding specificity was greatest for the guanine-rich sequences and RNAs with G-quadruplex (G4) secondary structures. Expression of constitutively closed ezrin rescued

the transcriptome profiles better than the constitutively open ezrin in an osteosarcoma cell line with its endogenous ezrin knocked out. Closed ezrin also rescued the metastatic capacity of osteosarcoma xenographs in zebrafish. Taken together, our data established that closed conformation of ezrin, which was previously considered inactive, can directly bind RNA and contributes to its metastatic phenotype.

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A Second-Generation XPO1 Inhibitor, Eltanexor, Demonstrates Potent Activity Against PAX3::FOXO1 Fusion Positive Rhabdomyosarcoma

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Background: Fusion-positive Rhabdomyosarcoma (FP-RMS) is driven by the oncogenic chimeric transcription factor PAX3::FOXO1 and has a dismal 5-year survival rate of 13% for metastatic patients. Thus, we hypothesized that targeting the fusion gene with small molecules would be an effective therapeutic strategy.

Study Design: We endogenously HiBiT-tagged PAX3::FOXO1 using CRISPR-cas9 in two FP-RMS cell lines, RH4 and SCMC. We performed a drug screen using the Mechanism Interrogation Plate library of 2,480 FDA-approved and preclinical compounds to identify those that downregulate PAX3::FOXO1 protein at 24 hours. Eltanexor, an XPO1 inhibitor, emerged as a top hit. XPO1 exports over 200 proteins from the nucleus by recognizing their nuclear export signal, including FOXO1, which is retained in PAX3::FOXO1. We thus hypothesized that eltanexor induces PAX3::FOXO1 nuclear accumulation, leading to cytotoxicity. Nuclear and cytoplasmic levels of PAX3::FOXO1 were measured by Western, and RNA-seq was performed to identify the transcriptomic impact of eltanexor. Furthermore, we tested the combination of eltanexor with other drugs that target FP-RMS.

Results: Eltanexor enhanced PAX3::FOXO1 nuclear accumulation at 2 hours in SCMC and 6 hours in RH4, followed by downregulation at 24 hours, leading to cell death. Two prominent mechanisms for apoptosis after XPO1 inhibition are p53 accumulation and impaired cell cycle gene export. By Western, eltanexor-treated SCMC cells demonstrated p53 nuclear accumulation at 6 hours after peak PAX3::FOXO1 nuclear accumulation, indicating that PAX3::FOXO1 could drive cytotoxicity. Eltanexor-treated SCMC cells also showed insignificant changes in cell cycle progression suggesting an alternate mechanism.

RNA-seq in RH4 cells demonstrated downregulation of PAX3::FOXO1 and MYCN signatures, components of the regulatory network in FP-RMS. Finally, we found that combining eltanexor with Mivebresib, a BRD4 inhibitor, demonstrated significant synergy.

Conclusions/Future Directions: Nuclear accumulation of PAX3::FOXO1 induced by eltanexor resulted in cytotoxicity for FP-RMS cells, which demonstrates the Goldilocks Principle, reported in Ewing's sarcoma, where too little or too much expression of fusion gene expression is detrimental to cancer cell survival. Thus, eltanexor offers a novel therapeutic approach for FP-RMS, and once validated, our findings could have significant translational implications for developing targeted therapies. Future experiments include total and phospho-proteomics, gene knockdown, and in vivo studies.

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A Tale of States: Decoding the Role of MYCN Amplification in ADRN-MES Plasticity

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Neuroblastoma (NB) is a lethal childhood cancer driven by chromosomal instability, with MYCN amplification (MNA) being the strongest prognostic marker. Despite initial therapeutic responses, high-risk NB frequently relapses, highlighting the importance of tumor plasticity in disease progression.

NB plasticity results from the dynamic interplay between Adrenergic (ADRN) and Mesenchymal (MES) states. While ADRN cells predominate in untreated tumors, MES cells enrich post-chemotherapy, driving resistance and relapse.

Despite increasing evidence of NB plasticity, key questions remain:

1) Do MES cells arise from ADRN-to-MES interconversion, or does therapy select a pre-existing, drug-resistant MES population?

2) What are the molecular and genetic mechanisms that drive NB state transitions?

To answer these, we tracked the fate of NB patient-derived xenograft models after therapy using in vivo single-cell barcoding sequencing, and profiled the genetic features associated with MES and ADRN states.

We demonstrate that MES cells exist pre-treatment as a rare population in vivo and expand after chemotherapy through both selection and interconversion. Barcode tracing confirms both mechanisms, revealing the persistence and accumulation of pre-existing barcoded MES cells after therapy, as well as ADRN cells undergoing MES reprogramming. Notably, relapsed tumors re-establish a high prevalence of ADRN cells, indicating that MES-to-ADRN interconversion occurs at relapse.

MYCN expression inversely correlates with the MES state, being absent or reduced in MES cells. Genetic profiling using spectral karyotyping and multiplexed FISH reveals that MNA loss correlates with MES transition. The MYCN gene amplicon undergoes a dynamic reorganization during cell-state transitions, shifting from extrachromosomal DNA (ecDNA) to chromosomal integration in homogeneously staining regions, or being lost entirely in some MES cells. Whether these changes arise from genetic drift, differential segregation of ecDNA, or active mechanisms of ecDNA expulsion/reintegration is under study.

Our findings reveal that NB resistance and relapse are driven by pre-existing heterogeneity and dynamic cell-state interconversions. We uncover the role of MNA dynamics in shaping tumor plasticity, highlighting its potential as a vulnerability. By revealing mechanisms of MYCN silencing and ecDNA dynamics, this work opens new avenues for targeting MES-associated plasticity and overcoming chemoresistance in high-risk NB.

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miR-21 Regulates T-Cell Alloreactivity and GVHD Severity in Experimental Allo-HCT

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Background: Graft-versus-host disease (GVHD), a major complication of allogeneic hematopoietic cell transplantation (allo-HCT), is driven by donor T-cell responses to host antigen-presenting cells. MicroRNA-21 (miR-21), a post-transcriptional regulator involved in immune modulation and oncogenesis, has been widely studied in cancer but remains poorly defined in the allo-HCT context. We hypothesized that miR-21 limits GVHD by modulating T-cell effector responses.

Methods: We utilized miR-21-deficient (miR-21−/−) donors in well-established murine models of GVHD and GVL. GVHD severity was assessed through clinical scoring, histopathology, and survival. T-cell activation and cytokine profiles were analyzed by multiparameter flow cytometry and Luminex assay on days +7 and +14 post-transplant. GVL was assessed in the FVB→BALB/c and B6→F1 systems using luciferase-labeled A20 (lymphoma) and P815 (mastocytoma) tumor cells, respectively. Bulk RNA-seq was performed on sorted donor CD4+, CD8+, and CD11b+ splenic populations.

Results: Littermate miR-21−/− and wild-type mice were immunophenotypically indistinguishable at baseline in spleen, lymph nodes, and thymus. However, recipients of miR-21−/− donor grafts experienced significantly more severe GVHD, evidenced by increased mortality, weight loss, elevated clinical GVHD scores, and severe histopathologic damage—most notably in the gut. These outcomes were accompanied by enhanced donor T-cell activation, increased proinflammatory cytokine production, and elevated cytotoxic markers, both at the cellular level and in recipient serum. Mixing studies revealed that this phenotype was driven primarily by miR-21 deficiency in donor T-cells (vs. myeloid cells). When complimented by HCT experiments using bm1 (MHC I mismatch; CD8-mediated GVHD) and bm12 (MHC II mismatch; CD4-mediated GVHD) recipients, miR-21 deficient CD4+ T-cells appear to be the primary drivers of systemic disease and mortality. Notably, miR-21−/− donor grafts demonstrated superior leukemia control in a syngeneic A20 lymphoma model, suggesting a potential role in modulating the balance between GVHD and GVL effects.

Significance: These findings identify miR-21 as a key regulator of donor T-cell alloreactivity and GVHD severity, particularly via CD4+ mechanisms. Bulk RNA-seq of sorted, donor-derived, CD4+, CD8+, and CD11b+ cells may define key dysregulated pathways. Targeting miR-21 may offer a strategy to reduce GVHD while preserving beneficial GVL, informing novel RNA-based therapeutic approaches in allo-HCT.

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Apheresis Product Characteristics Predict Response to CD22 CART-cells in Pediatric and Young Adult Patients with B-ALL

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Introduction: About 30% of patients receiving CART therapy for B-cell acute lymphoblastic leukemia (ALL) have primary non-response. (1,2]

Data on predictors of response, especially in pediatrics, are limited. Given the ability to optimize manufacturing and pre-collection therapies, identifying key biomarkers at the time of apheresis is imperative for improving outcomes. We hypothesized that T-cell fitness prior to CART manufacturing could distinguish responders from non-responders.

Methods: We retrospectively analyzed CD4/CD8-selected apheresis (T-cells) from children and young treated with CD22 CAR T-cells for B-ALL (NCT02315612). Analysis included flow cytometry for T-cell immunophenotype, activation, and exhaustion, Seahorse metabolic profiling, and RNA sequencing. We compared patients who achieved a complete response (CR) with those who had partial response, progressive, or stable disease (NR). Flow cytometry data was analyzed using a LASSO machine learning algorithm to develop a prognostic score.

Results: Across 30 patient apheresis samples, 22 (73%) achieved CR and 8 (27%) did not (NR). Demographics, with exception of disease burden, were comparable. Median pre-infusion disease burden was higher in patients with NR: 59% (r: 0.33-97.9) vs. 28% (r: 0.04-98.2%) in CR (p=0.24). Analysis of flow cytometry data by FlowSOM distinguished NR from CR by absence of activated, na"ive CD4 cells, and presence of CD4 effector cells. Manual gating confirmed that patients with NR had significantly lower CD4/CD8 ratios, fewer na"ive CD4 cells, lower expression of CD62L, CD28 and CD127, and higher CD69. RNA sequencing similarly showed lower CD127 expression in the NR group. The proportion of CD4 cells, CD4+CD127+, and CD4+CD69+ cells were selected in the predictive algorithm, and the resulting ROC curve had an area under the curve (AUC) of 0.96. Even among

patients with high baseline disease burden (M3 marrow, >25% blasts) (n=18), T-cells from those with NR (n=6) differed significantly from those with CR. Specifically, having <50% of CD4 cells expressing CD127 fully separated NR from CR in this cohort.

Conclusion: Our analysis shows that starting material characteristics can predict non-response to CART, using a flow cytometric assay alone. These assays can be integrated into routine apheresis processing, and the results can provide real-time guidance to patients, families, and providers.

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Successful Implementation of Mock Magnetic Resonance Imaging (MRI) in Children with Neurofibromatosis Type 1 Requiring General Anesthesia for MRIs

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Introduction: Magnetic Resonance Imaging (MRI) is frequently used in children with Neurofibromatosis type 1 (NF1) enrolled on clinical trials. General anesthesia (GA) is often required given the scan length and/or underlying neurocognitive disorders. MRIs under GA require pre- and post- safety evaluations, food restrictions, intravenous catheter insertion, and recovery time. Mock MRI helps prepare children for MRI exams and may reduce the need for GA.

Methods: We retrospectively collected data on all patients (pts) with NF1 who had a Mock MRI at the National Institutes of Health (3/2019-3/2024). Mock MRI sessions included assessments of the pts and families' needs, followed by an individual session using a non-functional MRI scanner and other age-appropriate educational preparatory materials. During the Mock MRI session, the Recreational Therapist taught coping and relaxation strategies to support the pts' ability to tolerate the procedure. Following the session a plan

for subsequent non-sedated MRI or additional supportive measures (e.g. repeat Mock MRI, oral anti-anxiety medication) was developed.

Results: Eleven pts with NF1 (age 8-24 years) participated in a Mock MRI session. Two pts had an underlying diagnosis of a neurocognitive disorder (ADHD n=1, ADHD and autism, n=1) requiring medication, two pts reported "anxiety" without a formal diagnosis, and two pts were noted by the provider to fidget and have difficulty sitting still during history and physical exam. After participation in at least one Mock MRI session, 10 pts (91%) successfully completed a clinically evaluable MRI without GA. Six pts received an oral anti-anxiety medication to facilitate the unsedated scan. Only one pt has required GA for an MRI obtained after the Mock MRI sessions.

Conclusion: Mock MRI sessions for pts with NF1 previously requiring GA for MRI scans were feasible and allowed most pts to perform subsequent MRIs without GA successfully. The ability to obtain high-quality, evaluable MRIs without GA in these pts has potentially improved safety, decreased cost, and increased efficiency. Based on our experience, Mock MRIs may be a useful tool in facilitating unsedated MRIs for children and young adults with NF1 and may be helpful in children with other conditions requiring repeated MRI scans.

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KAT2A and MYCN interact to drive oncogenic transcriptional regulation in neuroblastoma

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Background: MYCN is an oncogenic driver in neuroblastoma with limited therapeutic potential due to its structural disorder. MYCN relies on cofactors to regulate gene transcription, contributing to its oncogenicity; thus, cofactors are potential therapeutic targets. A MYCN interactome assay identified the cofactor KAT2A, a STAGA complex subunit with histone acetyltransferase activity, but their relationship hasn't been well-characterized.

Aim: Investigate KAT2A and MYCN cooperativity and determine if KAT2A is a potential therapeutic target

Methods: We utilized RNA-seq, chromatin immunoprecipitation sequencing (ChIP-seq), genetic knockdown, and therapeutic testing to evaluate KAT2A and MYCN interactions

Results: ChIP-seq results showed that MYCN knockdown in IMR32 cells resulted in a >30% decrease of KAT2A signals at MYCN peak centers, whereas MYCN overexpression in SHEP cells increased genome-wide binding of KAT2A. ChIP-seq analysis of MYCN and KAT2A overlapping peaks found that these sites were involved in protein translation and RNA processing, which are canonical MYC targets. MYCN can bind to the KAT2A promoter, and MYCN silencing in IMR32 resulted in a 40% decrease in KAT2A mRNA as well as a 14% decrease in KAT2A protein. KAT2A's involvement in MYCN protein stability was studied by silencing KAT2A, resulting in a 30-60% decrease of MYCN protein levels. HEK293T cells co-transfected with both MYCN and KAT2A or MYCN alone and treated with cycloheximide exhibited increased MYCN half-life from 34 minutes to 240 minutes when KAT2A was present. We used GSK983, a KAT2A/KAT2B PROTAC degrader, to target KAT2A. After 24hrs of treatment in three MYCN-amplified NB cell lines, there was a 35-80% depletion in MYCN protein levels as well as reduced cell proliferation and viability.

Conclusions: KAT2A and MYCN form a feed forward circuit where MYCN drives KAT2A transcription, recruits KAT2A to regulate gene transcription, and KAT2A stabilizes MYCN to drive malignancy. There's potential for KAT2A to serve as a therapeutic target.

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Angubindin-1-Mediated Disruption of Brain Endothelium Enhances T-cell Recruitment and Drug Delivery in Rodent Glioma Models

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The blood-brain barrier (BBB) limits effective drug entry, allowing malignant glioma progression. Previous studies demonstrated tricellular junction protein angulin-1 (lipolysis-stimulated lipoprotein receptor/LSR) inhibition enhanced brain delivery in non-tumor bearing rodents. Our studies evaluate the effect of LSR inhibition, with binding protein angubindin-1, on immune cell regulation and glioma progression. We first treated rat brain endothelial (RBMVEC), human-derived glioma (CBTN7316-6349 and LN-229) and rat glioma cells (S635) with angubindin-1 (75-600 µg/mL) to assess LSR protein interactions over 24 hours. S635 cells were treated with angubindin-1 (6-600 µg/mL) for 12-48 hours and then seeded into a 16-well electronic Boyden chamber to assess cell migration. Rat glioma models were administered doxil (3 mg/kg), angubindin-1 (10 mg/kg), or combination for 14 days and then monitored for toxicity, permeability and survival. We found that angubindin-1 dose-dependently increased LSR expression in endothelial and glioma cells, post 24 hours ($p<0.05$). Cellular fractionation confirmed angubindin-1 attached to membranous LSR, time-dependently decreased between 2-8 hours ($p<0.05$). Angubindin-1 enhanced cell

migration in S635 cells over a 36-hour period ($p<0.05$), with migration levels returning to baseline by 48 hours. In our immunocompromised LN-229 glioma models, treatment with angubindin-1, both alone and in combination, resulted in increased tumor migration without a survival benefit ($p<0.01$). In the immunocompetent S635 glioma models, angubindin-1 increased CD3+ T-cell recruitment in tumoral and peritumoral (± 250 mm from tumor bulk) regions compared to control, doxil, and combination treatments (166 vs 235 cells/mm2, $p<0.01$). Also, CD4+ T-cell recruitment in peritumoral region was higher in all treatment groups compared to control (134 vs 149 vs 185 cells/ mm2, $p<0.05$). Interestingly, angubindin-1 and combination treatment reduced CD8+ T-cell recruitment in the peritumoral region ($p<0.01$). No survival benefit was observed in immunodeficient S635 rat glioma models, suggesting increased CNS drug delivery must be paired with T-cell recruitment for favorable glioma effects from LSR inhibition. Ongoing studies exploring the role of LSR inhibition on immune cell trafficking CNS pharmacokinetics, and treatment resistance. Collectively, these studies explore the role of angubindin-1 as a potential additive agent for BBB disruption for improved CNS exposure, enhanced T-cell recruitment and prolonged survival.

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Metabolic profiling of MEKi-treated fusion-negative rhabdomyosarcoma cell lines suggests a role for glutamine antagonism as a preclinical therapeutic approach.

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Improved genetic characterization of rhabdomyosarcoma (RMS) subtypes, including PAX3/PAX7 fusion-negative RMS (FN-RMS) with RAS pathway mutations, has led to hope for individualized treatments with fewer side effects than conventional chemotherapy. Understanding the metabolic vulnerabilities of RMS remains an area with room for improvement. We hypothesized that determining the metabolic effects of RAS pathway inhibition in RAS-active RMS could inform new therapeutic combinations. We performed polar metabolomic profiling comparing trametinib and DMSO-treated FN-RMS cell lines, including two cell types with RAS mutations (SMS-CTR and RD) and one with an FGFR1 amplification (RMS-YM). Although inhibition of phospho-ERK was observed after 6 hours of treatment with trametinib in cells, limited effects on polar metabolites were observed at this time point. Following 24h of trametinib treatment, we observed alterations in purine and pyrimidine metabolism, amino acid metabolism, and central carbon metabolism. Based on these findings and our prior research on metabolic vulnerabilities other RAS-

active sarcomas, we investigated RMS cell dependence on glutamine, the most abundant amino acid in serum and a crucial metabolic contributor of nitrogen and carbon substrates to these pathways. FN-RMS cell lines demonstrated reduced colony formation in media with low glutamine. The clinical glutamine antagonist, DRP-104, inhibited the growth of RAS-RMS cells with IC50 values in the low micromolar range. We investigated the effects of DRP-104 on markers of tumor growth, DNA damage, and cell death by western blotting. As our own and other data support a role for MEKi and glutamine antagonism in RAS-active tumor models, future investigations will seek to combine these agents with other small molecule inhibitors suggested by metabolomics analyses in cell-based and in vivo RMS models.

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Developmental states determine intrinsic resistance to immunotherapy in pediatric rhabdomyosarcoma

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Background: Rhabdomyosarcoma (RMS) is an aggressive pediatric soft tissue sarcoma originating from skeletal muscle progenitors. Multiple immunotherapy clinical trials have found no evidence of immune reactivity against RMS, largely attributed to their relatively low mutation burden. We made an unexpected observation that RMS tumors had a spectrum of MHC-I expression, a key mechanism for anti-tumor activities of many immunotherapies. We hypothesized that the expression of antigen processing machinery (APM) is associated with myoblastic cell state, mimicking normal human embryonic myogenesis. In this study, we explore the etiology of antigen presentation in RMS and systematically evaluate epigenetic agents that sensitize RMS to immunotherapy.

Methods: Surface MHC-I expression was evaluated on pediatric tumor cell lines (n=65) from five pediatric tumor types. RNA-seq, whole exome and genome sequencing data were analyzed from RMS tumors (n=247) and iPSC models of skeletal muscle development (PMID: 32011235). Epigenetic drugs targeting DNA methylation (decitabine), EZH2 (tazemetostat), Class I/IV HDACs (mocetinostat), and LSD1 (GSK-LSD1) were used at sub-cytotoxic doses on RMS cell lines (n=10). Surface and total MHC-I expression were subsequently analyzed with flow cytometry and RNA-seq. Engineered human T cells

expressing a TCR recognizing an HLA-A*2 restricted peptide from PRAME were used in T cell cytotoxicity assays against RMS PDX cultures with epiDrug treatment.

Results: RMS cell lines, PDXs, and tumors exhibited a range of MHC-I expression, with most having low or undetectable surface expression. Exome sequencing of RMS tumors did not detect frequent genomic alterations in the APM pathway. The developmental state in RMS tumors was associated with the APM gene signature and surface MHC-I expression. Treatment of RMS cell line and PDX cultures with low-dose decitabine or mocetinostat restored surface MHC-I. Finally, targeting the RMS antigen PRAME using TCR therapy is enhanced by combination with epigenetic therapy.

Conclusions: We revealed that epigenetic suppression of APM is a new hallmark of RMS, mimicking embryonic skeletal muscle development. Epigenetic drugs targeting DNA methylation or class I/IV HDACs upregulated APM and enhance T cell toxicity against RMS. These findings may explain the lack of RMS responsiveness to immunotherapy and unlock new therapeutic strategies against RMS targeting MHC-I antigens.

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Dual-Targeting TCR-CAR T Cells for Enhanced Immunotherapy in High-Grade Gliomas

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Background: T-cell-based immunotherapies, including chimeric antigen receptor (CAR) T cells and antigen-specific T cell receptor (TCR) T cells, face significant challenges in treating high-grade gliomas (HGG), one of the most aggressive pediatric brain cancers. Both approaches have inherent limitations, including inconsistent T cell activation, poor persistence, and therapy-induced exhaustion. However, each platform also offers unique

advantages, and their integration may provide a synergistic strategy to enhance therapeutic efficacy and overcome these barriers. B7-H3, a pan-cancer antigen, and gp100, a melanoma-associated transmembrane glycoprotein, are overexpressed in HGG, making them promising immunotherapeutic targets. Here, we engineered dual-specific gp100-TCR and B7-H3 CAR T cells to leverage the complementary strengths of both platforms, aiming to mitigate antigen escape, enhance T cell persistence, and improve cytotoxic function.

Objective: This study evaluates whether co-expressing a gp100-specific TCR on B7-H3 CAR T cells enhances cytotoxicity and persistence against HGG.

Methods: T cells expressing the gp100 TCR were transduced with a B7-H3 CAR to generate dual-specific TCRgp100-B7H3.28.mutæ CAR (TCR-CAR) T cells. Functional analyses included repeated stimulation and MTS cytotoxicity assays to assess efficacy and persistence. Cytokine expression was measured by ELISA. Memory phenotyping and exhaustion marker expression (PD-1, TIM-3, and TIGIT) were evaluated using flow cytometry.

Results: TCR-CAR T cells demonstrated significantly enhanced persistence, proliferation, and sequential killing capacity compared to CAR-only T cells. While initial cytotoxicity was comparable between groups, TCR-CAR T cells maintained superior cytotoxicity at lower effector-to-target ratios during repeated stimulations, whereas CAR-only T cells exhibited a progressive decline in cytotoxic function. Enhanced persistence in TCR-CAR T cells correlated with increased IL-2 and IFNã production. Mechanistically, early findings suggest that TCR-CAR T cells undergo alterations in memory T cell phenotypes and epigenetic modifications in exhaustion-related pathways during repeated stimulation.

Conclusion: These findings support the integration of TCR and CAR platforms to enhance T cell function and persistence against brain tumors. Future studies will evaluate in vivo efficacy and further elucidate the molecular mechanisms underlying these effects, with the goal of optimizing adoptive T cell therapies for HGG.

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The in vivo dynamics of CD22 CAR T-cells: pharmacokinetic approach to cellular therapy monitoring

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Background: Pharmacokinetic data for chimeric antigen receptor (CAR) T-cell therapies have been limited due to complex dynamics of in vivo cell proliferation. Establishing patterns of CAR T-cell expansion and persistence is critical for predicting its safety and efficacy.

Objective: To describe the pharmacokinetics of CD22 CAR T-cells in relation to clinical response and baseline disease burden in children and young adults with relapsed/refractory B-cell acute lymphoblastic leukemia.

Design/Methods: CAR T-cell pharmacokinetics from patients who received 0.3e6 CAR T-cells/kg on an expansion cohort of a CD22 CAR T-cell trial were calculated from flow cytometry-based measures. Peripheral blood (PB) was collected weekly post-infusion and bone marrow (BM) was evaluated around Day +28 and as feasible until the time of alternative therapy (e.g. stem cell transplant).

Peak CAR T-cell concentration (Cmax), time of peak concentration (Tmax), and area under the curve (AUC) between Day 0-28 were calculated. Kaplan-Meier curves were generated to estimate CAR T-cell persistence in the PB and BM. Pharmacokinetics were compared by Day 28 disease response (complete response (CR); no complete response (NR)) and baseline disease burden (low < 5% and high >5% blasts in BM). Kruskal-Wallis, Mann-Whitney, and Mantel-Cox tests were used to compare pharmacokinetics between groups.

Results: 45 patients (34 CR and 11 NR) were included. Compared to patients with NR, those with CR had a higher AUC (median: 7476 cells/mcL vs. 527.5 cells/mcL, $p<0.0001$), Cmax (median: 52 cells/mcL vs. 3 cells/mcL, $p<0.001$), and longer median CAR T-cell persistence (142 days vs. 26 days, $p<0.001$). Similarly, patients with a CR had higher % CAR T-cells in the BM at Day 28 (52% vs. 3%, $p<0.005$) and longer median CAR T-cell persistence compared to NR (365 days vs. 91 days, $p<0.0001$). Tmax was not associated with response. Pharmacokinetics did not vary by baseline disease burden.

Conclusions: Pharmacokinetic analysis of CD22 CAR T-cells demonstrated greater CAR T-cell expansion and persistence in patients with CR. In contrast to prior studies of CD19 CAR T-cells, expansion kinetics did not vary by baseline disease burden. In future studies, a standardized approach to pharmacokinetics will facilitate comparisons between CD22 and other CAR T-cell constructs.

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Emerging Themes in the Perinatal and Early Medical History in a Cohort of Patients with MEN2B Followed at the National Institutes of Health

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Background: Multiple endocrine neoplasia type 2B (MEN2B) is a rare autosomal dominant cancer predisposition syndrome caused by a germline mutation of the rearranged during transfection (RET) proto-oncogene. MEN2B is associated with 100% risk of medullary thyroid carcinoma (MTC) and diagnosis is often made in the second decade of life when curative MTC treatment is rarely possible. The classic MEN2B phenotype is well-described but is not immediately evident during the first year of life. We hypothesized that features of the perinatal and developmental history may provide opportunities to consider an earlier diagnosis of MEN2B.

Methods: We analyzed available pregnancy, perinatal, and early medical history for 51 patients with MEN2B (germline RET p.M918T mutation) who were enrolled in one of two natural history studies ongoing at the National Institutes of Health (NCT01660984 or NCT03739827).

Results: Median age of MEN2B diagnosis was 10 years (interquartile range: 7-10, range: <1 – 30 years), and 28 (55%) were female. Complications during pregnancy and/or labor were reported in 24/48 (50%) individuals and included decreased fetal movements and placenta previa. Delivery by cesarean section was reported in 17/51 (33%) and 16/43 (37%) required NICU admission. Feeding problems during the neonatal period occurred in 24/50 (48%) patients and 19/43 (44%) had failure to thrive. As least one aspect of developmental delay was reported in 25/37 (68%), with gross motor delay most frequently reported. By three years of age, 42/49 (86%) reported gastrointestinal (GI) issues, most commonly constipation; 17/34 (50%) reported apneic and/or breath holding episodes; 15/40 (38%) had musculoskeletal (MSK) abnormalities, most commonly foot abnormalities; and 13/37 (35%) received genetics and/or neurology consultation.

Conclusion: Perinatal complications, developmental delay, and gastrointestinal, neurological, and/or musculoskeletal manifestations in early childhood were frequently reported in patients with MEN2B. Greater awareness and early recognition can help lead to earlier diagnosis of MEN2B and MEN2B-associated MTC.

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Chimeric cytokine receptors increase NK-cell functionality against AML

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Chimeric antigen receptor (CAR) T cell for acute myeloid leukemia (AML) have had discouraging clinical outcomes. As alternative effector cells, natural killer (NK) cells are being investigated. NK cells are innately cytotoxic and are safe in allogenic infusions.

However, NK cells have exhibited low persistence in clinical trials, limiting their durable efficacy. In order to increase NK-cell persistence, we have designed antigen-specific chimeric cytokine receptors (CCRs) based on the interleukin-15 receptor (IL 15R).

Artificial receptors were designed using sequences from either the native IL 15Rl3 chain or the common gamma chain (y) and anti-CD123 or anti-CD276 scFvs. Primary NK cells were expanded from PBMCs with irradiated feeder cells following T-cell depletion. NK cells were genetically engineered with retroviruses. CCR-chain expression was optimized using in silica modeling and fluorescent protein tags. Construct surface expression was detected by flow cytometry. Antigen-specific signaling of CCRs was evaluated by stimulating CCR-NK cells with immobilized rhCD123 and detecting phosphorylated STATS (pSTAT5) using western blot. Antigen-specific cytotoxicity was assessed in 18-hour bioluminescence assays with luciferase-expressing target cell lines. CCR-NK cell serial cytotoxicity and survival were evaluated using live-cell imaging over 10 days. The persistence of CCR-NK cells was evaluated in an AML xenograft model.

CCR chains are stably expressed. CCR single chain pSTAT5 is not detectable with rhCD123 stimulation and while NK cells expressing both receptors display tonic signaling, stimulation with rhCD123 results in a 2-fold increase in signal. NK cells expressing both CCR chains targeting either CD123 or CD276 display elevated cytotoxicity vs. various cancer cells lines compared to unmodified cells, likely influenced by tonic activation. Cytotoxicity is dependent on intact CCR signaling and antigen binding. Anti-CD123 CCR-NK cells have increased long-term survival, continue to kill through AML rechallenges, and kill parent AML more effectively than antigen-knockouts. CCR-NK cells maintained greater numbers than unmodified cells in mice.

NK cells expressing our IL 15R-based CCRs demonstrate antigen-specific signaling and cytotoxicity dependent on expression of both our chimeric IL 15Rl3 and chimeric y. CCR chains improve NK-cell functionality and survival in vitro and in vivo. The outcome of our work will be a persistent anti-AML NK-cell therapy intended for clinical translation.

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Cognitive evaluation following image-guided whole brain proton FLASH

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Purpose: Neurocognitive toxicity from radiation (RT) is one of the more devastating late effects for many childhood brain tumor patients. Though RT can improve survival in some tumors, RT is often delayed or omitted for young patients when receiving conventional dose-rate RT (≤6Gy per min, CONV) due to devastating effects on neurocognitive development. FLASH (ultra-high dose rate, >40Gy per second) has been shown to improve neurocognitive deficits with most studies treating animals with electron RT. Proton FLASH, which can treat deeper targets, is more appropriate for clinical translation and studies evaluating this are currently limited. We aimed to evaluate the effect of whole brain proton FLASH and CONV RT on neurocognition in juvenile mice using a novel image-guided proton RT workflow.

Methods: Juvenile 7-8 week old C5B7L/6 mice were treated with whole brain FLASH and CONV proton RT (n=12/group per sex) with 10 Gy CBCT was utilized for reproducible treatment planning. Neurocognitive assessment was performed using novel object recognition (NOR) test and discrimination index (DI) was calculated (DI = (time with novel object - time with familiar object)/ total time) at 6 weeks and 12 weeks post RT. Brain imaging is completed using 7T magnetic resonance scanner (MR) at 3 and 6 months post RT.

Results: Average doses delivered were 10.36±0.12 Gy at FLASH (56.0±1.5 Gy/s) and 10.17±0.05 Gy at CONV (0.55±0.01 Gy/s). Film measurements and strip ion chamber confirmed good agreement with prescribed values and Faraday cup. Thus far, 26 female mice (FLASH n=9, CONV n=10, SHAM n=7) have undergone NOR testing at 6 weeks and a subset of these (FLASH n=3, CONV n=3) have completed 12 week NOR testing. Preliminary data at both timepoints shows the median DI for FLASH is positive and median DI for CONV is negative. Cognitive testing for the entire cohort, including both sexes at 6 and 12 weeks post RT, is being analyzed and will be reported along with MRI findings. This provides evidence for use of a clinical proton device to deliver FLASH RT. Preliminary NOR data finds mice treated with FLASH have improved DI, indicative of better memory performance compared to CONV.

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Characterizing and Isolating a Senescent Stem-like Blast Subpopulation in Philadelphia Chromosome-like Acute Lymphoblastic Leukemia

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Philadelphia chromosome-like B-cell Acute Lymphoblastic Leukemia (Ph-like ALL) is a poor prognosis leukemia subtype characterized by heterogeneous genetic alterations that result in a kinase-activated gene expression pattern. Our group previously generated scRNA-seq data of Ph-like ALL PDX models treated in vivo with small molecule inhibitors and identified a subpopulation of blasts with high senescence and HSPC-like gene signature and upregulation of AP1 activity. We found that B-ALL patients with high proportions of this senescence-associated stem-like (SAS) leukemia population have worse overall and event-free survival (1). In this study, we hypothesize that the SAS subpopulation represents a resistant, relapse-fated population and endeavor to isolate and functionally characterize the SAS population in Ph-like ALL. Gene expression markers from scRNAseq of Ph-like-ALL patient samples were analyzed to identify differentially expressed genes in the SAS leukemia population compared to other blasts. Expression of each candidate gene/protein was checked in the Human Protein Atlas leukemia cell line database. The human Ph-like ALL cell lines MUTZ-5 and MHH-CALL-4, and the mixed phenotype leukemia (MPAL) cell line JIH-5 were analyzed using multiparametric flow cytometry. We performed drug combination treatments with the BCL-2 inhibitors venetoclax or navitoclax, and the JAK inhibitor ruxolitinib. Additionally, we performed cell viability assays and live-cell imaging time course experiments. We determined that five differentially expressed genes (CD34, CD44, CCR6, ITGB7, and GPR183) were candidates for identifying and isolating the SAS leukemia population due to their expression on the cell surface, high expression in the SAS population and low expression in the rest of the blasts. We identified a CCR6 and CD44 double positive population by flow-analysis that comprised 16% of the Ph-like ALL MHH-CALL-4 population that was effectively eliminated with ruxolitinib treatment. We isolated a novel senescent stem-like leukemia blast population by flow cytometry and characterize AP1 expression. Flow cytometric staining and analysis of cell lines identified a subpopulation that was nearly eradicated in Ph-like ALL cell lines in response to treatment with ruxolitinib. Initial surface staining shows the size of this population is variable between cell lines. Further studies are needed to phenotypically characterize the relapse potential of this population.

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Stag2-cohesin mediates 3d genome dynamics to regulate oncogenic transcription in neuroblastoma

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Neuroblastoma is characterized by abnormal transcriptional regulation driven by a network of core regulatory circuitry (CRC) transcription factors (TFs) and cell type-specific enhancer landscapes. Neuroblastoma arises due to defects in cellular differentiation, either through a failure to terminally differentiate or a reversion to a pluripotent-like state. While CRC TFs are known to bind cell type-specific enhancers and promoters to define the transcriptional landscape necessary for proper differentiation, their functional interplay with 3D nuclear architectural proteins, such as cohesin complexes (comprising SMC1A, SMC3, RAD21, and STAG1 or STAG2), remains largely unexplored. 3D genome organization plays a critical role in defining the cell type-specific transcriptional landscape by facilitating enhancer-promoter looping, which undergoes dynamic rewiring during differentiation. Understanding how CRC TFs collaborate with cohesin to establish and maintain neuroblastoma-specific transcriptional programs could provide valuable insights into the mechanisms underlying neuroblastoma pathogenesis and differentiation defects.

To investigate how STAG1- and STAG2-containing cohesin regulate enhancer-promoter interactions essential for MYCN target gene transcription in neuroblastoma, genome-wide RNA-seq and ChIP-seq analyses were performed. While STAG1 knockdown (KD) had no impact on neuroblastoma cell proliferation, STAG2 KD was essential for cell viability. Genome-wide ChIP-seq revealed that STAG2-cohesin peaks were enriched at active promoters and enhancers of the neuroblastoma genome and colocalized with CRC TFs (MYCN, HAND2, GATA3, PHOX2). Although STAG1 KD did not alter cohesin occupancy at active promoters and enhancers, STAG2 KD led to a marked reduction in cohesin binding at these regulatory elements. Despite steady-state protein levels of CRC TFs remaining unchanged upon STAG2 KD, chromatin-bound MYCN was significantly diminished. ChIP-seq analysis further demonstrated that MYCN-bound active promoters and enhancers lost MYCN occupancy after STAG2 KD, which was accompanied by a loss of the active histone mark H3K27ac.

These findings underscore the critical role of STAG2-cohesin in regulating and maintaining the NB oncogenic transcriptome by selectively modulating MYCN chromatin binding. Importantly, this study highlights STAG2-cohesin's functional specialization, establishing it as a pivotal regulator of cohesin dynamics and neuroblastoma transcriptional programs. By demonstrating how STAG2-cohesin selectively facilitates MYCN binding to its target genes, this study provides a potential therapeutic avenue for targeting MYCN-driven transcription through the regulation of cohesin dynamics.

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Longitudinal Follow-Up and Outcomes of Pediatric and Adult Patients with SDH-deficient GIST

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Introduction: Gastrointestinal stromal tumor (GIST) are gastrointestinal non-epithelial neoplasms most commonly driven by somatic mutations in KIT or PDGFRA. Approximately ten percent of GIST are due to germline mutations in SDHx or epigenetic loss of expression of SDHC. Effective systemic therapies for SDH-deficient GIST have not yet been identified. A better understanding of the natural history of patients with these neoplasms is critical to identify effective treatments.

Methods: Data from patients with SDH-deficient GIST enrolled in a study from 2008 through August 2023 at the NIH were evaluated. (NCT03739827, NCT01109394, NCT00001686). The data collected came from both medical records of patients' visits to NIH as well as home providers. When available, tumors were characterized by sequencing

of SDH genes. Germline analysis of SDH genes was also offered to consenting patients and families.

Results: Clinical information and specimens were collected from 113 GIST patients (median age at diagnosis 26, [range 7-63] years; 71% (80) female, 29% (33) male) were classified by molecular subtypes: 29.2% SDHC epimutation, 34.5% SDHA, 22.1% SDHB, and 14.2% SDHC. Most commonly, patients had one surgery (54%) while a lower proportion had three or more (16.8%). Primary tumors occurred in the stomach with 33.6% having metastases at presentation the most common location being the liver (27.4%). Overall, 54% of patients were treated with imatinib, 40.37% were treated with sunitinib and 21.2% received other additional systemic therapies.

Conclusions: The data indicates that the patients in our cohort with SDHB deficiency have an increased mortality rate, while SDHC Epimutant have the lowest mortality rate. The most common SDH subtype to have metastases after presentation was SDHB with 76%. Patients with SDHB subtype also had the highest percentage of 3 or more surgeries at 28%. Across all 4 SDH deficient GIST categories, the majority of our patient cohort was treated with imatinib. The indolent disease nature of SDH-deficient GIST and medication toxicity intolerance makes it difficult to assess treatment effect. The participation of patients in natural history studies such as ours is paramount to ensuring that we are able to continue learning about this disease process and identify and design new therapeutic strategies.

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Endogenous HiBiT-tagging of PAX3::FOXO1 reveals that CDK inhibitors downregulate the fusion oncogene and disrupt core regulatory transcription in PAX3::FOXO1 positive rhabdomyosarcoma

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BACKGROUND: Oncogenic fusion genes are attractive therapeutic targets due to their tumor-specific expression. PAX3::FOXO1 (P3F) is the dominant oncogenic driver of fusion-positive rhabdomyosarcoma (FP-RMS) with no current targeted therapy.

METHOD: HiBiT-tag, an 11 amino acid peptide of NanoLuc luciferase, was inserted into the endogenous P3F using CRISPR and validated by Western blotting. RNA-seq and ChIP-seq were used to assess transcriptomics and DNA binding of HiBiT-tagged P3F (P3F-HiBiT). High-throughput drug screen was performed using the Mechanism Interrogation PlatE drug library with known mechanisms of action. Mouse xenograft models were used to investigate in vivo efficacy.

RESULTS: We validated the HiBiT tagging of P3F by Western. Both P3F-HiBiT and unmodified P3F activated FP-RMS gene sets in fibroblasts. ChIP-seq using HiBiT antibody verified that P3F-HiBiT binds to the same sites as P3F. A screen for drugs that downregulate P3F in both RH4 and SCMC identified 182 compounds. Drug classes found included HDAC inhibitors, BRD4 inhibitors, and CDK inhibitors. FP-RMS was most sensitive to CDK7, CDK9, and multi-CDK inhibitors. TG02, a multi-CDK inhibitor currently in human trials, showed the most significant downregulation of NanoLuc luciferase. Treatment with TG02 showed marked suppression of P3F targets by RNA-seq, and Western blotting confirmed the downregulation of P3F and core regulatory transcription factors, with evidence for inhibition of CDK9 with decreased RNA Pol2 Ser2 phosphorylation (Pol2S2). ChIP-seq for RNA Pol2 showed a decrease in transcription pause-release. Moreover, analysis of genes ranked by decreased Pol2S2 in the gene body showed significant enrichment for P3F targets ($p<0.001$). TG02 significantly delayed tumor progression without weight loss in four mouse xenograft and PDX models of FP-RMS.

CONCLUSION: By HiBiT-tagging P3F, we identified 182 drugs that suppress P3F levels. TG02, a top hit, showed in vivo efficacy, indicating that FP-RMS is susceptible to multi-CDK inhibition. Decreased occupancy of Pol2S2 in the gene body of P3F targets suggests that the mechanism of TG02 is primarily through transcriptional inhibition of P3F and the core regulatory transcription factors. This indicates that TG02 may effectively treat FP-RMS and other transcriptionally addicted cancers. Combination studies with standard-of-care chemotherapies are currently being tested for future translation into clinical trials.

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SETD2 Mediates EWS::FLI1 Tolerance in Non-Ewing Sarcoma Cells

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Ewing Sarcoma (ES) is an aggressive tumor that develops primarily in the bone or soft tissue in adolescents and young adults with poor prognosis for patients with recurrent and metastatic disease. ES is characterized by the presence of a tumor-specific chromosomal translocation resulting in the oncogenic fusion protein EWS::FLI1 that is present in 85% of the cases. EWS::FLI1 is the genetic driver mutation in ES and it is critical for ES initiation and maintenance. Moreover, EWS::FLI1 is required by ES cell lines and xenografts for survival as perturbation of EWS::FLI1 function results in growth arrest. However, EWS::FLI1 expression in non-ES cells is not tolerated, marked by induction of growth arrest, senescence or apoptosis, and no mouse model for ES exists since ubiquitous expression of EWS::FLI1 leads to embryonic lethality. Thus, tolerance of EWS::FLI1 expression and consequently induction of tumorigenesis requires a yet unknown permissive cellular state.

To identify genes and pathways that when inactivated provide EWS::FLI1 tolerance in non-ES cells, we performed a genome wide CRISPR-Cas9 KO screen in HEK293 cells. We discovered that the loss of the lysine methyltransferase SETD2 resulted in EWS::FLI1 tolerance in HEK293 cells. SETD2 KD in HEK293 cells resulted in improved EWS::FLI1 tolerance compared to cells expressing SETD2. While the loss of SETD2 did not alter EWS::FLI1 protein or mRNA levels in HEK293 or ES cells, we observed an interaction between SETD2 and EWS::FLI1 in ES cells via nuclear co-IP. This protein-protein interaction may provide a molecular mechanism of how SETD2 and EWS::FLI1 crosstalk in ES. How SETD2 is possibly altering EWS::FLI1 function or loss of SETD2 may create an epigenetic state in ES that provides EWS::FLI1 tolerance are currently under investigation.

The long term goal of our study is to understand the yet unknown molecular basis of early stage ES biology in which a permissive cellular state is created that allows cells to tolerate EWS::FLI1 expression which drives transformation. This may result in establishing clinically relevant experimental models and ultimately pave the way to develop novel treatment strategies against ES.

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BCL-6 corepressor-DNMT1 complex modulates IGF-1 transcription in retinoblastoma

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Background: BCL-6 corepressor (BCOR) loss-of-function alterations are common in clinically aggressive retinoblastoma. In our previous study, we found that BCOR loss promotes the growth and dissemination of retinoblastoma cells, and IGF-1 is highly upregulated in BCOR-mutant retinoblastoma. These findings suggest that targeting the IGF-1/IGF-1R signaling pathway significantly enhances anticancer effects in BCOR-mutant retinoblastoma cells. This study aimed to elucidate the mechanism by which BCOR mutations regulate IGF-1 transcription.

Methods: Gain- and loss-of-function strategies were used to modulate BCOR levels in retinoblastoma cell lines. Western blotting assessed H3K27 trimethylation, H3K36 dimethylation, DNMT1, BCOR, and actin expression. Pyrosequencing analyzed IGF-1 promoter methylation. Co-immunoprecipitation identified BCOR-DNMT1 interaction. Real-time PCR measured IGF-1 mRNA expression.

Results: In baseline retinoblastoma cell lines, the BCOR-high-expressing cell line WERI-RB1 showed a high methylation status at H3K27 and H3K36. BCOR knockout and knockdown significantly decreased methylation at H3K27 and H3K36, while BCOR overexpression increased methylation in retinoblastoma cells. These results suggest that BCOR regulates DNA methylation. Next, as the promoter region of IGF-1 contains three distinct methylation sites that regulate its transcription, we hypothesized that BCOR may regulate the methylation of the IGF-1 promoter region. To test this, we assessed these three methylation sites using pyrosequencing. Our results showed that methylation levels at the P2 and P3 sites were significantly decreased, leading to a substantial increase in IGF-1 mRNA expression in BCOR-knockout WERI-RB1 cells compared to wild-type BCOR WERI-RB1 cells. Since BCOR is a component of the PRC1.1 complex and does not regulate DNA methylation solely, we hypothesized that BCOR interacts with DNMT1, a DNA methyltransferase, to regulate methylation at the IGF-1 promoter region. Co-IP experiments confirmed that BCOR interacts with DNMT1. Next, we treated these cells with GSK-3484862, a DNMT1 inhibitor. DNMT1 inhibition sensitized only BCOR wild-type WERI-RB1 cells, suggesting that the BCOR-DNMT1 complex mediates methylation at the P2 and P3 sites of IGF-1, thereby inhibiting IGF-1 transcription.

Therefore, we have elucidated how BCOR regulates IGF-1 transcription in retinoblastoma.

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Combining MEK and CDK4/6 Inhibition: Synergistic Treatment of ATRT with Mirdametinib and Abemaciclib

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Background:Atypical teratoid/rhabdoid tumors (ATRT) is a poor-prognosis infantile brain tumor. Mirdametinib is a highly specific MEK1/2 inhibitor that has been reported to penetrate the blood-brain barrier better than other MEK inhibitors. Our previous study showed that mirdametinib inhibited ATRT growth in vitro, while its in vivo efficacy varied for different orthotopic xenograft models. Abemaciclib, a selective inhibitor of CDK4 and CDK6, is effective in blocking S phase entry. We hypothesize that a synergistic effect can be achieved by co-administering MEK inhibitor and CDK4/6 inhibitior.

Methods: Mirdametinib and abemaciclib effects were assessed through western blot for MAPK markers and cell cycle markers. Western blot analysis, Muse flow cytometer assays, and immunofluorescence were used for proliferation, apoptosis and synergy study. Mirdametinib impact on survival was determined in orthotopic xenograft models of ATRT.

Results: Mirdametinib and abemaciclib combination treatment achieved more proliferation suppression and apoptosis induction compared to single agent treatment in ATRT cell lines (western blot: pRb and cPARP). ATRT treated with combination therapy exhibited a higher level of cell cycle inhibition (Muse cycle assay, BrdU immunofluorescence staining: t-test, Growth Curve). Syngergy studies revealed a combinatorial effect of mirdametinib and abemaciclib treatment in CHLA06 and BT37 cell lines.

Conclusions: Mirdametinib and abemaciclib combination treatment showed enhanced efficacy compared to single agent. Our studies suggest that pharmacologically inhibiting the MAPK pathway and CDK4/6 may be an effective strategy to target ATRT and help extend survival in this deadly disease.

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Lineage-Specific Transcription Factor Screen Identifies TFs That Regulate Neuroblastoma Plasticity and Drug Resistance

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Keywords: Neuroblastoma, transcription factors, genetic screening, phenotypic switching, single-cell RNA sequencing

Background: Neuroblastoma (NB) exhibits intratumoral heterogeneity, with adrenergic (ADRN) and mesenchymal (MES) cell types capable of interconversion. Distinct transcription factor (TF) networks underpin these phenotypes, influencing their drug sensitivity (ADRN) or resistance (MES). However, it remains unclear whether each of these TFs drive ADRN and MES interconversion.

Aims: This study aims to identify TFs driving ADRN<->MES transitions in NB and their role in chemoresistance. We hypothesize that targeting these TFs can reveal mechanisms of plasticity and strategies to overcome therapy resistance.

Methods: We created two 10x Genomics-compatible barcoded TF libraries: 23 TFs from ADRN lineage signature genes and 29 TFs from MES lineage genes. The ADRN TF library was introduced into the MES-type SHEP cells, while the MES TF library was delivered into the ADRN-type SY5Y cells using doxycycline-inducible lentiviral vectors. Cells were treated with or without chemotherapeutic agents to apply selective pressure. Next-generation sequencing identified barcoded TFs enriched or lost after treatment, and single-cell RNA sequencing analyzed associated cell phenotype.

Results: DNA barcode sequencing analysis revealed that enrichment of TF read counts associated with chemotherapeutic resistance in ADRN-type cells were associated with 6/23 MES TFs. PRRX1, a known driver of the ADRN->MES transition supports the robustness of our screen. We identified SIX1 as a novel ADRN->MES reprogramming TF reprogrammed the mRNA landscape from ADRN toward a MES state and increased their resistance to cytotoxic therapies in vitro. Conversely, 3/29 reduced TF barcode read counts associated with increased chemotherapy sensitivity in MES-type cells. We hypothesize that the expression of these ADRN TFs in MES-type cells could reprogram the mRNA landscape from MES->ADRN.

Conclusions: This study identifies key TFs driving ADRN<->MES transitions and therapy resistance in NB, highlighting SIX1 as a novel driver of MES transitions. These findings provide a foundation for targeted therapeutic strategies to inhibit MES transitions and overcome drug resistance in NB.

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Deciphering the code of MYCN protein in neuroblastoma: unveiling its secrets through barcoded mutagenesis screen and single-cell RNA sequencing analysis

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Keywords: Neuroblastoma, MYCN, barcoded mutagenesis, single-cell RNA sequencing, oncogenic activity

Background: Amplification of the MYCN gene in neuroblastoma (NB) is linked to poor prognosis. MYC oncoproteins are key drivers of cancer but their mechanisms remain unclear, as they can drive tumorigenesis through gene transcription regulation, while recent studies suggest they may also promote tumorigenesis independently of gene expression changes. Since MYC functions depend on specific protein interactions, a structure-function analysis of MYCN is critical to identify therapeutic vulnerabilities in MYC-driven cancers.

Aim: To identify and dissect the functional regions of MYCN that drive its oncogenic and transcriptional activities in NB.

Methods: We generated a 10x Genomics-compatible barcoded library of MYCN mutants with 15 amino acid (AA) deletions spanning the 464-residue protein, including alanine scanning of the MBII domain. These constructs were introduced into SHEP cells, each carrying a single vector. Colony formation assays, tumorigenicity tests in mice, and barcode sequencing identified mutants with reduced oncogenic activity. Single-cell RNA sequencing and bioinformatic analyses revealed transcriptional changes and critical MYCN regions. Stable clones of selected mutants were created for further studies.

Results: Deletion of MYCN's MBI and MBII domains reduced both oncogenic and transcriptional activities, similar to c-Myc. Deletions in C-terminal regions containing DNA- and MAX-binding domains abolished transcriptional activity, with most also losing oncogenic activity. Alanine scanning of MBII identified key residues affecting activity: one

mutant lost both transcriptional and oncogenic functions, while others retained oncogenic activity despite reduced transcriptional activity. An intrinsically disordered region essential for transcriptional but not oncogenic activity was identified. Novel MYCN mutants with altered oncogenic activity showed changes in protein stability and target gene regulation, likely due to disrupted interactions with cofactors.

Conclusion: This study defines critical MYCN domains and residues driving oncogenic and transcriptional activities, providing insights into MYCN structure-function relationship in NB. Further analysis of mutant interactomes will uncover druggable cofactors, advancing therapeutic strategies for MYCN-driven cancers.

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Metastatic PDX models of Ewing Sarcoma: Development and Characterization

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Background: Although improvements in local therapy have increased the 5-year survival rate for localized Ewing sarcoma (EWS) from less than 20 % to 70 %, little progress has been made in the treatment of metastatic disease, which has a 5-year survival rate of less than 30 %. A major challenge in the field is a lack of preclinical models that can recapitulate spontaneous metastatic disease and can be used to better understand the disease and identify new vulnerabilities. In this study, we present and characterize a model of spontaneous distant EWS metastasis that mimics the clinical development of the human disease in different immune backgrounds mice. Methods: A panel of eight molecularly diverse patient-derived xenograft (PDX) models (SJ18, SJ17, S049, NCH1,

NCH4, PDMR-098, PDMR-077) were injected orthotopically into the gastrocnemius muscle in the left hind leg of athymic nude and NOD SCID gamma (NSG) mice. Once primary tumors reached 1500 cubic centimeter, we proceeded with hind limb amputation survival surgery and observed for the development of distant spontaneous metastases.

Results: EWS PDXs formed spontaneous metastases in multiple sites including lymph nodes, lung, liver, and kidney in both NSG and nude mice. Each PDX model exhibited a distinct pattern of metastasis formation, based on site and metastasis frequency. The highest frequency of distant metastases was seen in SJ18, SJ17, and PDMR-098 models

(85.71 %; 66.67 %; 63.64 % respectively). SJ18 most frequently metastasized to lung, whereas SJ17 and PDMR-098 most frequently metastasized to liver and mesenteric lymph

nodes. In NSG versus nude mice, PDXs showed variations in metastasis frequency and site preference with NSG mice demonstrating a higher rate of metastasis.

Conclusion: Here we describe a preclinical model of spontaneous distant EWS metastasis that recapitulates the characteristics of human disease. The site and frequency of metastases varies based on the PDX model and mouse background, highlighting that metastasis formation is a multifactorial process driven by complex interactions between the tumor and host. Future studies will focus on characterization of the metastatic tissue, compared to the primary tissue, with the goal of identification of novel targets for EWS metastases.

POSTER SESSION B

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Dual CAR T-Cell Therapy Targeting B7-H3 and CD123 Remodels the Tumor Microenvironment in Pediatric Glioma

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Background: Tumor-associated macrophages (TAMs) comprise about 30–50% of the tumor mass in pediatric high-grade gliomas (pHGG), where they contribute to an immunosuppressive tumor microenvironment (TME) that limits the effectiveness of adoptive T-cell therapies and promotes T-cell exhaustion. CD123 (IL-3 receptor alpha) is expressed on a subset of M2-like TAMs, making it a potential therapeutic target. We hypothesize that targeting CD123 to deplete immunosuppressive TAMs could enhance the anti-tumor activity of B7-H3 CAR T cells in pediatric glioma models.

Objective: The primary objective of this study was to evaluate whether a dual CAR T-cell strategy targeting both B7-H3 and CD123 can augment anti-tumor immune responses by modulating the TME and overcoming TAM-mediated immunosuppression.

Methods: Immunocompetent pHGG models were employed to investigate the efficacy of anti-B7-H3 and anti-CD123 CAR T cells. Standard cytotoxicity assays, repeat stimulation experiments, and quadruple co-culture systems were utilized to assess the impact of B7-H3 CAR T cells both alone and in combination with macrophages (M0, M1, M2 phenotypes), with or without CD123 CAR T cells. In vivo, intratumoral administration of CD123 CAR T cells was used to evaluate their ability to deplete TAMs and assess the subsequent effects on the efficacy of B7-H3 CAR T cells. Tumor immune profiles and TME remodeling were assessed by high-dimensional flow cytometry.

Results: CD123 CAR T cells alone demonstrated minimal cytotoxicity against B7-H3+ glioma cells but were highly effective in depleting M2-polarized TAMs. In co-culture assays, the presence of macrophages suppressed the functional activity of B7-H3 CAR T cells, but this suppression was reversed upon the addition of CD123 CAR T cells, restoring CAR T-cell cytotoxicity. In vivo, administration of CD123 CAR T cells resulted in a significant reduction in TAM infiltration, particularly in M2-polarized clusters within the tumor, and led to a marked decrease in monocyte- and microglia-derived myeloid populations within 7–14

days following treatment, without inducing significant toxicity or adverse effects. Ongoing investigations are exploring whether the myeloid remodeling induced by CD123 CAR T cells enhances the therapeutic efficacy of B7-H3 CAR T cells.

Conclusion: These findings suggest that targeting CD123-expressing TAMs could represent a promising strategy to enhance the efficacy of CAR T-cell therapies by modulating the TME and overcoming the immunosuppressive barriers that hinder the effectiveness of CAR T-cell therapies in pHGG.

Keywords: CAR T-cell therapy, Diffuse Intrinsic Pontine Glioma (DIPG), Tumor-associated macrophages (TAMs), Tumor microenvironment (TME), Dual targeting, Macrophage depletion

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Acquired resistance to MEK inhibition alters glutamine metabolism and promotes therapeutic vulnerability to the glutamine amidotransferase inhibitor DRP-104 in NF1-associated malignant peripheral nerve sheath tumor

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The interactions between oncogenic signaling pathways and metabolism are incompletely understood in pediatric and adolescent/young adult sarcomas. Patients with the cancer predisposition syndrome neurofibromatosis type I (NF1), in which tumors have activated RAS-MEK-ERK signaling, are at risk for the aggressive sarcoma malignant peripheral nerve sheath tumor (MPNST), the most common cause of death in people with NF1 under age 40. Our prior research showed that that preclinical MPNST models are sensitive to small molecule inhibition of glutamine amidotransferases (GA), primarily though effects on nucleotide metabolism. The goal of this project was to identify how MEK-ERK signaling impacts glutamine-dependent purine synthesis in NF1-associated tumors. These investigations are clinically relevant as many patients with NF1-MPNST may have been previously treated for other NF1 tumors with FDA approved MEK inhibitors (MEKi). We hypothesized that MEK-ERK signaling promotes glutamine utilization for purine synthesis, and resistance to MEKi may alter these pathways, leading to differential sensitivity to GA inhibition. To test this hypothesis, we took advantage of paired MEKi-sensitive parental (ST8814-P) and MEKi-resistant (ST8814-R) MPNST cells that have been previously characterized and found to have increased HGF/MET signaling in the resistant cells. We examined glutamine nitrogen use for purine and pyrimidine biosynthesis between parental

and resistant cells by LC-MS isotope labeled flux analysis using 15N1-glutamine-amide. These studies found that ST8814-R cells had decreased enrichment of 15N in guanosine monophosphate (GMP) at 12 hours compared to ST8814-P cells (42% m+3 GMP in S8814-P compared to 29% m+3 GMP in ST8814-R), suggesting decreased glutamine metabolism by amidotransferases in de novo purine synthesis (DNPS) in the setting of acquired MEKi resistance. MEKi resistant cells were more sensitive to inhibition of GA by DRP-104 with IC50 values at least 2-fold lower than parental cells (6.4 μM in ST8814-P versus 3.3 μM in ST8814-R at 72h), suggesting that reduced glutamine utilization as a nitrogen source in DNPS confers sensitivity to a GA inhibitor. One potential reason for these observations may be if MPNST cells rely on MEK-driven pathways to regulate DNPS, and this regulation is altered upon development of MEKi resistance. Of note, Western blotting did not demonstrate an effect of MEKi on total protein levels of selected purine synthesis enzymes including PFAS, IMPDH, GMPS, HPRT1. Future experiments will address whether post-translational regulation of purine biosynthesis is altered upon development of MEKi resistance in MPNST or other NF1-associated tumors such as glioma. We will also investigate markers of nucleic acid damage upon treatment with DRP-104 and investigate combination HGF/MET inhibitors with DRP-104, in order to better target altered glutamine metabolism in MEKi resistant MPNST.

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Advancing NF1 research through a comprehensive biorepository of primary tumor specimens, preclinical models, genomic and clinical data for NF1-associated tumors

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Purpose: Neurofibromatosis type 1 (NF1) is an inherited neurocutaneous condition associated with increased risk of developing cutaneous neurofibromas (cNF), plexiform neurofibromas (pNF), atypical neurofibromatous neoplasms of uncertain biologic potential

(ANNUBP), and malignant peripheral nerve sheath tumors (MPNST). The Johns Hopkins NF1 Biospecimen Repository, established in 2016, provides essential resources for NF1 research via access to primary human tissues and preclinical models. It has since become a vital resource for NF1 research worldwide and continues to expand the scope of available biospecimens to address growing requests from the NF research community. In addition to the growing collection of banked tumor tissue, the biorepository has broadened its offerings to include single-cell suspensions from tumors, tissue microarrays (TMA), genetically diverse cell lines, and patient-derived xenografts (PDX).

Methods: The NF1 biorepository includes clinically and genomically-annotated samples from patients with NF1, including blood fractions and tumor tissues. Tissue samples are frozen, paraffin-embedded, and digested into single-cell suspensions. Cell lines and PDX are attempted with malignant tumors. New cell lines are evaluated with IncuCyte live-cell imaging to assess growth and response to MEK and SHP2 inhibitors. Banked specimens are genomically analyzed via whole exome sequencing (WES), whole genome sequencing (WGS), and RNA sequencing (RNAseq), with data accessible through the NF Data Portal. Clinical annotations and outcomes data are made available to investigators upon scientific review and IRB approval.

Results: Over 400 unique samples have been banked from 206 patients, including pNF (n=93), MPNST (n=70), cNF (n=124), blood fractions, and xenograft (n=5) specimens. RNAseq (n=89), WES (n=114), and WGS data (n=21) are available through the NF Data Portal. Three novel patient-derived MPNST cell lines sensitive to SHP2 or MEK inhibition have been generated. A new TMA panel of cNF, pNF, MPNST, and control tissues has been validated with key biomarkers and is available upon request. To date, 83 research requests have been granted access to specimens and data.

Conclusions: The Johns Hopkins NF1 Biospecimen Repository serves as a high-quality, clinically and genomically characterized resource to NF1 research, continually evolving to meet scientific needs by expanding its specimen offerings.

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Combined SHP2 and CDK4/6 inhibition depletes intratumoral tumor-associated macrophages in malignant peripheral nerve sheath tumors

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Purpose: Malignant peripheral nerve sheath tumor (MPNST) tumorigenesis is driven by the loss of the tumor suppressor protein, neurofibromin, which leads to hyperactivation of RAS effector pathways, suggesting that targeting of RAS-ERK signaling could be effective. We previously reported that the combination of SHP2 and CDK4/6 inhibitors had antitumor activity in immunocompromised preclinical models of MPNST. It is unknown, however, whether the presence of intratumoral immune cells may enhance the efficacy of these targeted therapies or how the therapies impact tumor immunity. Furthermore, there is emerging evidence regarding the immunomodulatory effects of molecularly targeted therapies, suggesting that immunotherapy-based combinations may provide more durable responses. Our studies of human MPNST have revealed that pathological, intratumoral myeloid cells contribute to immune surveillance evasion. Utilizing small molecule inhibitors holds the potential to directly target tumor cells as well as alter the immunosuppressive tumor microenvironment, thereby enhancing responses to immunotherapies when used in novel combinations. We therefore aim to determine the interaction between molecularly targeted therapies, with SHP2 and CDK4/6 inhibitors, and the tumor immunobiology in a preclinical model of MPNST.

Methods: We used an immunocompetent, syngeneic NF1-/-;Ink4a/Arf-/- mouse model of MPNST to investigate the tumor immune microenvironment (TIME) interactions with targeted inhibitors. Tumor-bearing mice received treatment with TNO155 (SHP2i), ribociclib (CDK4/6i), and their combination. We extracted treated tumors for analysis via immunohistochemistry and multiparameter flow cytometry to immunophenotype the intratumoral immune cells.

Results: The combination of SHP2 and CDK4/6 inhibition had profound anti-tumor efficacy in the syngeneic mouse model. Treatment with TNO155 resulted in depletion of tumor-associated macrophages (TAM) and enrichment of CD8+ T cells infiltrating the tumor. The addition of ribociclib further diminished TAM populations. Furthermore, TNO155 treatment depleted intratumoral myeloid-derived suppressor cells (MDSC), but the addition of ribociclib did not further modulate this compartment.

Conclusions: SHP2 and CDK4/6 inhibitors lead to alterations in the TIME. The immunomodulatory effects of SHP2 inhibitors provide rationale for combinations with immune checkpoint inhibitors. Further investigations on the adaptive signaling changes within the TIME following exposure to targeted agents can aid in designing novel combinations in the treatment of MPNST.

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Evaluating the role of splicing in Ewing Sarcoma using an SRRM2-GFP knock-in experimental system

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Introduction: The oncogenic driver of Ewing Sarcoma, EWS::FLI1, has a critical role in mRNA splicing, which occurs in biomolecular condensates including nuclear speckles. This led us to create a system to image and investigate SRRM2, which functions as the nuclear speckle scaffold.

Methods: ES type 3 (A4573) cells were used. SRRM2-GFP knock-in was completed using CRISPR-Cas9 delivered via transfection. Following, cells underwent puromycin selection. Live cells were plated on 35mm glass-bottom dishes. Fluorescence recovery after photobleaching (FRAP) data was captured using NIS-Elements. Time course imaging was completed over 6 hours in 10-minute intervals. Fixed cells were grown on glass coverslips. Cells were fixed with 4% Formaldehyde and stained with SRRM2 polyclonal primary and secondary antibodies and imaged with optimal z-stack for 3-D localization. Pearson's value of colocalization (PVC) describes how closely two proteins may be interacting. A PVC nearing 1 indicates a strong positive correlation.

Results: Imaging reveals that SRRM2 appears as puncta localized to the nucleus of ES cells. In static images, cells undergoing mitosis showed dissolution of SRRM2 labeled bodies. FRAP enables us to evaluate the mobility of speckles by measuring the rate and extent of fluorescence recovery of a protein following photobleaching. A series of FRAP experiments demonstrated a bleach in GFP fluorescence to 18.4-44.8% followed by a recovery of 63.8-71.1%. SRRM2-GFP was imaged in combination with a nuclear speckle antibody (SRRM2). Colocalization of cropped ROIs of speckles from SRRM2-GFP and antibody PVC range (0.766-0.954) ($N=25$, $p<0.005$) was measured.

Discussion: Static images validated SRRM2 tagging through colocalization analysis and indicated that SRRM2 bodies dissolved during mitosis. FRAP data demonstrated SRRM2 mobility in Ewing cells, which supports their biophysical characteristic of forming biomolecular condensates. This leads us to believe that nuclear speckles contribute to splicing condensate organization, in support of other literature. Irregularities in fluorescence for some FRAP curves may be due to cellular movement that displaced ROIs.

Conclusion: This work validates the SRRM2-GFP CRISPR model and indicates that the composition and activity of condensates and splicing proteins may have important impacts upon splicing often observed in cancer.

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Engineered T cell therapy in combination with TGF-beta signaling blockade for immunotherapy against osteosarcoma

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Background: Osteosarcoma is an aggressive bone sarcoma with only a 60% 5-year survival rate despite the use of intensive multimodal therapy. Complex genomic abnormalities characteristic of osteosarcoma create inter- and intra-patient variability, which has slowed the application of targeted therapies. However, osteosarcomas have high immune cell infiltration indicative of mutation-associated immunogenicity, although immunotherapies have been largely ineffective to date. We recently identified TGF-beta, a pleiotropic immune-suppressive growth factor, as being expressed higher in osteosarcoma than any other human cancer. The etiology of this expression and the therapeutic relevance to immunotherapy are unknown.

Study Design & Results: In this study, we evaluated a clinically relevant adoptive TCR therapy targeting the cancer testis antigen PRAME against osteosarcoma cell line and patient-derived xenograft mouse models. We found that co-transduction of T cells with a dominant-negative TGFBR2 (dnTGFBR2) dramatically improved the response rate to adoptive T cell therapy, with multiple mice achieving complete responses. Immunophenotypic profiling of tumor infiltrating lymphocytes (TIL) from osteosarcoma tumor models demonstrated significant expansion of dnTGFBR2 expressing T cells and favorable markers of T cell activation. To explore the mechanisms underlying the improvement in T cell therapy, PDX tumor-infiltrating lymphocytes (TIL) were isolated after 10 days and profiled using RNA-seq, ATAC-seq, and single cell RNA-seq with CITE-seq of T cell phenotyping markers (n=16). PRAME-TCR T cells co-expressing dnTGFBR2 had a 50% reduction in dysfunctional terminally exhausted CD8+ T cells (P=.003), doubled the effector memory CD8+ T cells (P=.0067), and tripled the central memory T cells (P=.0046)

when compared to PRAME-TCR T cells alone. These changes were associated with a 30-fold increase in global chromatin accessibility, possibly related to a more favorable T cell state.

Conclusions and Significance: In summary, the efficacy of adoptive T cell therapy against osteosarcoma was greatly improved by co-expressing dnTGFBR2 on engineered T cells. Protecting T cells from chronic exposure to high levels of TGF-beta, such as by co-expressing dnTGFBR2, has considerable potential in unlocking anti-tumor immune responses in osteosarcoma and other cancers. This construct will be further developed for clinical trials in patients with cancers expressing PRAME and TGF-beta.

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Multiplex Staining and VINE-seq Demonstrates the Temporospatial Complexity of the Blood-Tumor-Barrier in Pediatric-Type Diffuse High Grade Glioma

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Pediatric-type high-grade gliomas (HGGs) are aggressive brain tumors characterized by rapid growth, infiltrative behavior, and significant therapy resistance. One major challenge in treating HGGs involves the interplay of tumor cells to their microenvironment; allowing for creation of the blood-tumor barrier (BTB). Our studies combined histologic and transcriptomic evaluations of immune-deficient rodent injected with H3K27M mutant pediatric-type glioma cells. Multiplex staining provided temporospatial analysis of known cellular markers involved in BBB formation, including known pericyte, tight-junction, and immune markers. Regions of interest were classified as tumor core, peritumor (+250μM), or distal (>250μM). Analysis was conducted with the use of QuPath and cell segmentation

performed using Mesmer, with expression proportions quantified for each cell/protein marker. Utilization of vessel isolation and nuclei extraction for sc-RNAseq (VINE-seq) explored the molecular landscape of vasculature within the tumor microenvironment. All rodent models were untreated, evaluated early to late post-intracranial injection (D20-50). Preliminary results revealed elevated endothelial tight junctions within distal regions compared to the core ($p<0.0001$), demonstrating an intact BTB furthest from tumor bulk. Within the tumor core, high Iba1+ macrophage (46%) and low neuronal (1%) expression was present ($p<0.0001$), while highest localized expression of glioma cells, and PDGFRβ+ pericytes were present among intratumoral core vasculature; all at late disease progression ($p<0.0001$). Initial VINE-seq studies identified varied cell-types in early disease models and ongoing studies will identify regional and temporal expression changes. These findings offer cellular and molecular HGG microenvironment changes over time; which underscores the importance of neuronal-immune interactions related to the restrictive BTB. Future studies are aimed at identifying genomic and proteomic expression patterns, to gain a better understanding of pediatric-type HGG progression for predictions of improved treatment timing.

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ROME, a novel membrane protein that accelerates Ewing sarcoma metastasis

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Less than 25% of Ewing sarcoma (ES) patients with metastatic disease live beyond five years and there are no effective therapies available to specifically target metastatic disease. Through an in vivo genome-wide CRISPR/Cas9 transcriptional activation screen using human ES cells as xenografts in zebrafish, we identified the human INAFM2 gene as a strong driver of metastasis and therefore named the vertebrate gene and its protein product, ROME (Regulator of Metastasis). Up until now, INAFM2 has not been studied in-depth and INAFM2 protein expression profile or function is not known.

We characterized aspects of ROME expression and function in vertebrate development, physiology, and pathology. Blocking ROME expression in zebrafish embryos resulted in severe developmental defects and early mortality. Single cell RNA sequencing of zebrafish embryos with rome knockdown and RNA sequencing of cancer cell lines with ROME modulation revealed that ROME negatively regulates calcium and canonical Wnt pathways. We also discovered that ROME directly interacts with CAV1, FLOT1, and Vimentin proteins. ROME overexpression increased metastasis of ES xenografts in zebrafish and

immunodeficient mice while ROME knockout in ES xenografts reduced metastasis. In summary, we discovered a previously unstudied human glycoprotein located on the plasma membrane that can regulate ES cell motility and invasion and increases the metastatic phenotype of ES tumor cells in mouse models.

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CRISPR-Drug Combinatorial Screening Identifies Effective Combination Treatments for MTAP null cancer

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CDKN2A/MTAP co-deletion occurs frequently in non-small cell lung cancer and other solid tumors including glioblastoma and pancreatic ductal adenocarcinoma. Lung cancer remains the leading cause of cancer-related mortality, and fewer than 15% of glioblastoma or pancreatic cancer patients survive 5 years, underscoring the need for more effective therapies1–5. PRMT5 is a synthetic-lethal dependency in MTAP null tumors and an attractive therapeutic target for CDKN2A/MTAP deleted cancers. A new revolutionary class of inhibitors, referred to as MTA-cooperative PRMT5 inhibitors, has shown promising results in ongoing early phase clinical trials. Nonetheless, effective cancer treatment typically requires therapeutic combinations to improve response rates and defeat emergent resistant clones. Thus, we sought to determine whether perturbation of other pathways could improve the efficacy of MTA-cooperative PRMT5 inhibitors. Using a paralog and single gene targeting CRISPR library we screened MTAP deleted cancers in the presence or absence of MTA-cooperative PRMT5 inhibitors. We identified several genes sensitizing to PRMT5 inhibition including members of the MAP kinase pathway. We demonstrate that chemical inhibition of MAP kinase pathway members using KRAS, MEK, ERK, and RAF inhibitors synergize with PRMT5 inhibition to kill CDKN2A/MTAP null, RAS-active tumors. Further, MTA-cooperative PRMT5 inhibitors combined with either KRAS or RAF inhibitors led to in vivo complete responses, emphasizing the potential benefit for patients. Lastly, we demonstrate that cell lines resistant to KRAS inhibitor are not resistant to MTA-cooperative PRMT5 inhibitors and vice versa, suggesting non-cross-reactive mechanisms of resistance.

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Pan-RAS inhibition as a novel therapeutic strategy in RAS-driven rhabdomyosarcoma

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Dysregulated RAS signaling represents a therapeutic vulnerability for the majority of PAX-fusion negative rhabdomyosarcoma (FN-RMS). One third of FN-RMS cases have oncogenic driver mutations in HRAS, NRAS and KRAS. An additional proportion have receptor tyrosine kinase (RTK) amplifications that hyperactive RAS signaling. Mutant RAS drives rhabdomyogenesis, impairs myogenic differentiation and maintains chemotherapy refractory tumor cells. Despite the frequency of these alterations, no RAS-targeted therapies have proven clinically viable for RAS-mutant RMS. Epigenetic variables may attenuate the efficacy of inhibitors of RAS. Among the genes identified as recurrently altered in RMS is BCOR (BCL-6 corepressor), a component of the PRC1.1 complex. Loss of function BCOR mutations occur in 15% of FN-RMS, and frequently co-occur with RAS-pathway alterations. The potential cooperative effects of hyperactive RAS and loss of BCOR in RMS have not been studied. Further, the effects of BCOR mutations on the antitumor properties of RAS-directed therapies has not been elucidated.

Pan-RAS inhibitors (pan-RASi) are a novel class of therapeutics that target oncogenic and wild-type (WT) RAS isoforms and inhibit the binding of RAS effectors, including RAF and PI3K. The efficacy of pan-RASi has been demonstrated in RAS-mutant PDAC and NSCLC, and RMC-6236 is being evaluated in an early-phase clinical trial (NCT05379985). Its utility in RAS-driven RMS has not been determined. We therefore set forth to evaluate the efficacy of pan-RAS in a genomically heterogeneous panel of RAS-driven RMS cell lines. We found that pan-RASi reduced RAS-effector signaling in FN and fusion-positive (FP)-RMS cell lines. Specifically, pERK was inhibited up to 72 hours in our sensitive cell lines. Pan-RASi impaired cell growth at nanomolar concentrations in short-term and long-term 2D growth assays. Tumor colony formation in 3D growth assays was also prevented with pan-RASi. However, BCOR-mutant RMS cell lines showed decreased growth inhibition in response to

pan-RASi compared to BCOR-WT cell lines. Thus, further studies are needed to delineate the mechanisms driving reduced inhibitor efficacy in the setting of BCOR mutations. Our preliminary studies demonstrate the efficacy of pan-RASi in RAS-driven in vitro RMS models. If proven effective in vivo, pan-RASi could translate into clinical trials for patients with RAS-driven RMS

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Disparities between bone marrow B-cell aplasia and minimal residual disease in pediatric and AYA B-ALL after CAR T-cell therapy

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Background: CAR-T cell therapy use in B-ALL is well-established. While flow cytometry based minimal residual disease (FC-MRD) testing is standard for monitoring B-ALL, next generation sequencing (NGS-MRD) using the Adaptive clonoSEQ® platform is becoming widely utilized due to increased sensitivity in detecting residual leukemia. Additionally, peripheral blood (PB) B-cell aplasia (BCA) as a surrogate for CAR-T cell persistence is increasingly being explored to predict post CAR-T relapse. Data regarding bone marrow (BM) BCA using NGS (NGS-BCA) is limited with no prior comparison to BM BCA by flow cytometry (FC-BCA) or NGS-MRD.

Objective: To cross-compare NGS-MRD, NGS-BCA, BM FC-BCA in patients who achieved a BM FC-MRD negative complete remission (CR) on day +28 following CAR-T therapy.

Design/Method: Single institution retrospective study of patients who received CD22 or bivalent CD19/22 investigational CAR-T therapy at the National Cancer Institute from 5/2018 until 4/2024. Patients were assessed for NGS-MRD and NGS-BCA at approximately day +28 post-CAR. BM NGS-BCA was defined as <1% B-cells.

Results: Of the 26 patients achieving FC-MRD negative CR, 7 received CD22 CAR, and 19 received bivalent CD19/22 CAR. The median age was 15 years (range 4-38). Fifteen subjects (58%) were NGS-MRD positive. Of the 11 NGS-MRD negative patients, 10 (91%)

had NGS-BCA. The majority, 11 of 15 (73%) patients, with NGS-MRD positivity also had NGS-BCA. Nineteen (79%) of 24 evaluable patients had BM FC-BCA. Two patients (8%) had NGS-BCA but not FC-BCA; one patient had FC-BCA without NGS-BCA. Thirteen of 26 patients (50%) relapsed; 77% (10/13) had NGS-BCA and 62% (8/13) were NGS-MRD positive at day +28. All 8 patients with pre-CAR extramedullary disease (EMD) relapsed; this included 4 patients who were NGS-MRD negative. Across 10 patients with both NGS-MRD negativity and NGS-BCA at day +28, 4 (40%) relapsed. All 22 patients with available PB counts demonstrated post-CAR PB FC-BCA.

Conclusion: The majority of patients with FC-MRD negativity achieved marrow NGS-BCA and FC-BCA, neither of which was fully concordant with NGS-MRD at day +28. Importantly, the majority of patients who relapsed post-CAR had NGS-MRD positivity on day +28 or a history of EMD, regardless of BCA.

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Genome-wide CRISPR-Cas9 screening to interrogate mechanisms of CAR T-cell resistance in neuroblastoma

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Despite intensive multimodal regimens, more than 50% of patients with high-risk neuroblastoma succumb to late relapses due to resistant minimal residual disease (MRD). Chimeric antigen receptor (CAR) T-cells present a promising therapeutic modality to target MRD and improve survival. Nevertheless, while clinical success has been observed in subsets of hematological malignancies, CAR T-cell therapy has proven less effective in treating solid tumors in part due to physical barriers, antigen heterogeneity, and the immunosuppressive tumor microenvironment (TME). We hypothesize that the identification of key tumor-intrinsic mechanisms that confer resistance to CAR T-cell therapy is critical to improving treatment efficacy and survival of children with high-risk neuroblastoma. To this end, we employed a genome-wide CRISPR-Cas9 knockout screen using the human Brunello pooled single guide RNA (sgRNA) library in neuroblastoma cells. Briefly, genetically diverse tumor cells [IMR-5 (MYCN-amplified, p53-WT), SH-SY5Y (MYCN-

WT, p53-WT) and SK-N-FI (MYCN-WT, p53-M246R)] expressing Cas9 were transduced with Brunello lentivirus and subjected to untransduced mock T-cells or B7-H3, GD2, or GPC2 CAR T-cells in vitro. Surviving tumor cells were collected, and genomic DNA was extracted. Enriched sgRNAs were detected by Illumina sequencing and analyzed using the MAGeCK algorithm. Top hits were selected and further validated with a focused library which showed that sgRNAs targeting genes involved in GPI-anchor biosynthesis and attachment, post-translational modifications, and transcriptional regulation were significantly enriched in surviving CAR T-cell-treated neuroblastoma cells compared to those treated with untransduced mock T-cells. Among these, we identified loss of PIG/PGAP, SPPL3, STAT1, STAT3, or MYCN as potential regulators of CAR T-cell response in neuroblastoma. Single-gene validation studies demonstrate that loss of PIG/PGAP and SPPL3 induce changes in antigen levels, suggesting antigen downregulation as mechanism of resistance. On the contrary, loss of the transcription factors STAT1, STAT3, or MYCN induces global changes in the neuroblastoma cell transcriptome and renders resistance through modulation of the T-cell response. Our findings provide additional evidence suggesting that antigen-dependent and antigen-independent mechanisms contribute to CAR T-cell therapy resistance in neuroblastoma.

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Secretome distinguishes spectrum of NF1 associated peripheral nerve sheath tumor

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Purpose: Early detection and interception of malignant transformation in Neurofibromatosis 1 (NF1) associated peripheral nerve sheath tumors (PNST) are challenging due to insensitive clinical symptoms, limited specificity of standard of care imaging, and the limited negative predictive value of invasive tissue biopsy. Novel non-invasive and tumor site-agnostic surveillance assays provide potential for early diagnosis and intervention, especially during the critical transformation from benign plexiform neurofibromas (PN) to pre-malignant atypical neurofibromas (AN). We hypothesize that circulating proteins in the plasma (the secretome) accurately distinguish the spectrum of PN, AN and malignant peripheral nerve sheath tumors (MPNST).

Methods: The secretome of 118 plasma samples (Healthy n = 10, PN n = 29, AN n = 25, and MPNST n = 54) from 79 patients were analyzed using a proximity extension assay (PEA) panel for 1461 proteins (Olink). Unique protein signatures for each tumor state were

identified using one-versus-all comparisons: Healthy-versus-all (HvA), PN-versus-all (PvA), AN-versus-all (AvA), and MPNST-versus-all (MvA) with ANOVA and post-hoc Tukey honestly significant difference (HSD) of normalized protein expression (NPX) outputs from PEA. Proteins considered significant had a NPX difference ≥ 1.2 and p adj <0.5. Individual protein's performances were assessed using Youden's index and a receiver operating characteristic (ROC) curve.

Results: 88 proteins were significant for HvA comparisons, 114 proteins for PvA, 136 proteins for AvA, and 284 for MvA. Individual proteins' performance for HvA had a median AUC of 0.76 (interquartile range (IQR): 0.72-0.8), median sensitivity 0.60 (IQR: 0.53-0.81), median specificity 0.90 (IQR: 0.8-1.0); PvA had a median AUC 0.64 (IQR: 0.62-0.67), median sensitivity 0.38 (IQR: 0.33-0.47) , and median specificity 0.97 (IQR: 0.86-1.0); AvA had a median AUC 0.70 (IQR: 0.67-0.73), median sensitivity 0.52 (IQR: 0.44-0.61) and median specificity 0.84 (IQR: 0.76-0.92); MvA had a median AUC 0.76 (IQR: 0.73-0.79), median sensitivity 0.57 (IQR: 0.52-0.67), and median specificity 0.92 (IQR: 0.84-0.96).

Conclusions: This pilot demonstrates that circulating proteomics non-invasively distinguish between disease states in NF1. Importantly, PEA uses just 40uL of plasma per sample enabling the integration of non-invasive orthogonal biomarkers, such as cell free DNA, all from one tube of blood.

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FHD-286, a small molecule inhibitor of the SWI/SNF ATPases SMARCA2/4 blocks neuroblastoma phenotypic switching

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Background: Neuroblastoma (NB) exhibits two primary cellular phenotypes: the majority of NB tumor cells are adrenergic (ADRN) and sensitive to cytotoxic drugs, while a minority are mesenchymal (MES) and resistant. The ability for NB tumors to switch phenotypes contributes to their aggressiveness, chemotherapy resistance, and recurrence. Mechanisms controlling phenotypic switching are ill-defined. One possible mechanism is via SWI/SNF ATPases (SMARCA2/4), which are members of chromatin remodeling complexes that remodel nucleosomes to regulate lineage specific gene expression during development.

Aims: To determine whether targeting SWI/SNF ATPases can reduce NB cell plasticity and sensitize cells to chemotherapeutic drugs.

Methods: FHD-286, a small molecule inhibitor of SMARCA2/4 currently in clinical trials, was evaluated for its effects on the growth of five NB cell lines (SHEP, SY5Y, IMR32, BE2C, IMR5) alone or in combination with etoposide. The ability of FHD-286 to alter NB plasticity was assessed using multiome snRNA-seq analysis of a model in which PDX-derived NB neurospheres switch to MES and ADRN phenotypes in standard culture media within 2-3 days.

Results: FHD-286 inhibits growth of the four NB cell lines with ADRN features (IC50=20-138nM) while the one MES cell line was relatively unaffected. The depletion of SMARCA2/4 using a PROTAC degrader (AU-15330) also inhibits growth of ADRN-type NB cell lines, but it is less effective (IC50=43-1344nM). FHD-286 synergizes with etoposide in SY5Y and IMR32 cells. PDX-derived MAST97 NB neurospheres develop ADRN and MES phenotypes and their respective gene signatures when cultured in standard media. However, the presence of FHD-286 (400nM) prevents these neurospheres from adopting distinct MES and ADRN phenotypes when shifted to standard media.

Conclusions: These findings establish FHD-286 as a novel therapeutic strategy for inhibiting NB phenotypic switching and as a combination treatment with cytotoxic drugs to prevent tumor resistance.

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A novel human mesenchymal stem cell model of Ewing sarcoma recapitulates EWS::FLI1 associated protein interactome involved in mRNA splicing and chromatin remodeling BAF complex.

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Introduction: Connections between epigenetic reprogramming and transcription or splicing create novel mechanistic networks that can be targeted with tailored therapies. EWS::FLI1 is the most common initiating event in Ewing sarcoma (ES), the second most common bone malignancy in children and young adults. EWS::FLI1 is intrinsically disordered oncoprotein and their protein binding partners is necessary for their oncogenic function.

We previously showed EWS::FLI1 strongly associated with proteins involved in mRNA splicing, RNA processing and chromatin remodeling in ES. In order to recreate the EWS::FLI1 associated protein complexes in preclinical model system, we chose to develop an oncological model of ES based on its proposed cell of origin, the human mesenchymal stem cell (hMSC).

Methods: Since hMSC are not tolerant to long-term EWS::FLI1 expression, we first disrupt the function of p53 and Rb by stably expressing the Human Papilloma Virus (HPV) E6 and E7 proteins. In order to assist cellular immortalization, we also induced expression of Human Telomerase Reverse Transcriptase (hTERT), which is commonly expressed in ES. These modified hMSC (expressing E6/E7 and hTERT) were then transformed by stably expressing the proximity labeling mini-turbo (MT) fused full-length EWSR1, FLI1 or EWS::FLI1 into these modified hMSC to study the EWS::FLI1 proteomic interactions. Biotinylated proteins are identified through mass spectrometry, and scored using a statistical tool, SAINT analysis, to define high-confident proximal interactions.

Results: We found the known EWS::FLI1 interacting protein partners involved in the mRNA splicing (DDX5, HNRNPK, SFPQ) and Chromatin remodeling BAF complex (ARID1A, SMARCA4, SMARCB1). These protein interactors are strongly validated our hMSC model recapitulate the ES phenotype of EWS::FLI1 oncogenic functional interactome. In addition, we found newly described unique protein (DLX5, HOXA9, TRA2B) interactors within the EWS::FLI1 specific proximity interactome.

Conclusion: This validated unique hMSC Model can now be used for dissection of epigenetic alterations, transcription, and alternative splicing that are driven by EWS::FLI1 through its protein partners.

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Molecular Mechanisms of PROTAC-Induced Target Degradation in Fusion-Positive Rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children and adolescents. RMS can be classified as fusion-positive (FP-RMS) or fusion-negative (FN-RMS), based on the presence of PAX3::FOXO1 or PAX7::FOXO1 fusion proteins. Chromosomal translocations cause the formation of chimeric oncogenic fusion proteins where the N-terminal DNA binding domain of PAX3 or PAX7 fuses to the C-terminal

transactivation domain of FOXO1. Patients with FPARMS have worse event free survival rates and there are no targeted therapies focusing on fusion proteins yet. Currently, most RMS patients are treated with a multi-modal approach including surgery, radiation and chemotherapy. Unfortunately, these treatments options do not provide desired outcomes when RMS tumors metastasize, acquire resistance, or relapse. In an effort to develop novel targeted therapies, we designed proteolysis-targeting chimeras (PROTACs) for the PAX3::FOXO1 fusion protein. PROTACs are bifunctional molecules comprising of three segments: a target protein ligand, a linker region and a E3 ligase ligand, which eventually degrade target proteins by ubiquitinating the protein of interest. We performed a small molecule library screen to identify compounds that can directly bind to recombinant PAX3::FOXO1 protein. We synthesized 15 new PROTACs targeting PAX3::FOXO1 by conjugating small molecule hits from our initial screen with a known E3 ligase. We started evaluating the PROTAC-induced PAX3::FOXO1 degradation in FP-RMS cell lines (Rh30, Rh41, CW9019, U48484 and U66788).

We are also assessing how effectively PROTACs enter the cells by evaluating the intrinsic fluorescence of the hit compounds. Our preliminary data identified at least one promising PROTAC that can reduce PAX3::FOXO1 protein levels. Our findings have the potential to provide new treatment options for RMS patients and their improved survival and quality of life.

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Determinants of chemotherapeutic resistance in fusion-positive rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma, and is classified as fusion-positive (FP) or fusion-negative (FN) based on the presence of the PAX3::FOXO1 or PAX7::FOXO1 fusions. These chimeric proteins are formed by reciprocal chromosomal translocations and consist of an N-terminal portion of the PAX3 or PAX7 transcription factor fused to a C-terminal portion of the FOXO1 transcription factor. FP status is associated with worse event free survival for patients with RMS. Regardless of fusion status, most RMS patients receive a chemotherapy regimen consisting of some combination of vincristine, actinomycin D, and cyclophosphamide (VAC). Unfortunately, tumors may acquire resistance to these drugs over time, leading to relapse. There are

common drug resistance pathways among cancers that involve cells exporting the drugs, modifying the drugs or altering drug targets. Our work aimed to uncover novel genes that drive VAC resistance in FP-RMS using a two-pronged approach. To assess how FP-RMS cells gradually acquire resistance, we cultured FP-RMS cell lines (RH30, RH41, CW9019, U48484, U66788) in sublethal concentrations of vincristine, actinomycin D, or 4-hydroperoxy cyclophosphamide (4-HC, the active metabolite of cyclophosphamide) for multiple passages. We periodically assessed the IC50 of cells growing in drugs and increased drug doses as cells developed resistance. We generated five cell lines with a ~5- to ~20-fold increase in IC50. Using RNAseq, we identified differentially expressed genes between the parental and resistant cell lines that may act as drivers of chemotherapeutic resistance. To assess de novo resistance in FP-RMS, we used CRISPR inactivation and activation library screening. We generated RH30 cells stably expressing Cas9 or dCas9-VPR, an endonuclease-dead version of Cas9 fused to three transcriptional activators. We then transduced these cells with a CRISPR inactivation sgRNA library (18,885 gene targets) or a CRISPR activation sgRNA library (19,113 gene targets). Transduced cells were cultured in vincristine, actinomycin D, or 4-HC, and surviving cells were expanded and sequenced to determine which genes provided drug resistance when activated or deleted. We will present the results of the RNAseq analysis from acquired resistance studies and the CRISPR library screening findings from the de novo resistance experiment.

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Long-term hematologic effects of selumetinib treatment in children with inoperable plexiform neurofibromas

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Purpose: Selumetinib is the first FDA approved treatment for children with neurofibromatosis type 1 (NF1)-associated plexiform neurofibromas. In a phase 1/2 clinical trial (SPRINT, NCT01362803), selumetinib was associated with mild, reversible cytopenias.

Prolonged treatment is needed for durable tumor response, so it is important to understand potential long-term adverse effects. This study characterizes hematologic changes with long-term use of selumetinib in children with NF1.

Methods: Data obtained from the SPRINT study database. Comprehensive blood count (CBC) with differentials collected per protocol at baseline and before cycles (1 cycle=28 days). Enrollment began in 2011 and data was collected through 2/7/2025. CBCs obtained during drug holds were included. White blood cell count (WBC, k/μL), hemoglobin (Hgb, g/dL), platelets (Plts, k/μL) and absolute neutrophil count (ANC, cells/μL) were analyzed. Simple linear regressions and descriptive statistics were calculated with GraphPad Prism version 10.4.1.

Results: There were 98 participants (59 male, median age at enrollment 10.6 years, range 3-18.5) treated for a median 67.1 cycles per person (range 2.7 to 143.5) with a total 2597 CBCs (median 24 per participant, range 3-78). Across all CBCs, the WBC, ANC, Hgb, and Plts were within the normal range 91%, 88%, 77% and 91% of the time, respectively. The minimum values were WBC 2.02 K/μL, ANC 690 cells/μL, Hgb 6.9 g/dL, and Plts 95 k/μL. No participants required a dose reduction or drug hold for a hematologic adverse event. There was no statistically significant change over time for 73% (n=72), 87% (n=85), 59% (n=58) and 74% (n=73) of participants for WBC, ANC, Hgb and Plts, respectively. For those with a statistically significant change ($p<0.05$), the slopes for each parameter included both positive and negative values with no clear trends over time.

Conclusion: The data reported here is the longest follow-up to date for pediatric patients with NF1 on selumetinib. No evidence of clinically meaningful changes in hematologic parameters was observed. Though small statistically significant changes were seen in some participants, there was no clear trend in any parameter. When values were below normal, decreases were transient and often associated with intercurrent illness. Additional analyses are ongoing.

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The Methylcytosine Dioxygenase Tet2 influences outcomes of Graft-versus-Host Disease through regulation of Donor T-cells

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Allogeneic hematopoietic stem cell transplantation (HSCT) is an essential component of therapeutic strategies for patients with hematological malignancies, and oftentimes the only curative treatment for aggressive childhood leukemias. However, graft-versus-host disease (GVHD) can be a significant complication, contributing to unacceptably high rates of mortality and morbidity. In contrast, graft-versus-leukemia (GVL) activity is a major benefit conferred from HSCT. Donor T-cells are the primary cellular mediators in both GVHD and GVL effects, with their activation status being highly dependent on their interactions with host antigen-presenting cells. These interactions and local inflammatory milieu induce epigenetic modifications within T-cells, such as DNA methylation (by DNA methyltransferases) or active de-methylation (by TET enzymes), resulting in altered T-cell function. Indeed, clinical data suggest that donor mutations in epigenetic modifiers such as DNMT3A or TET2, can result in higher risk of GVHD but also decreased risk of relapse and improved survival. We aim to understand the role of TET2 in donor T-cell responses following HSCT and specifically to describe its function in the GVH and GVL context, in order to identify therapeutically targetable pathways.

In our preliminary work, we utilized conditional T-cell specific TET2 knock-out (KO) mice (Cd4Cre+; Tet2flox/flox) as donors, in well-established, clinically relevant models of HSCT. Recipient mice that received donor T-cells lacking TET2 experienced significantly more severe GVHD, resulting in worse overall survival, relative to both syngeneically transplanted control mice and allogeneic controls receiving wild-type (WT) T-cells. These results suggest a critical role for DNA de-methylation by TET2 in regulating T-cell activation post-HSCT. Importantly, TET2-KO recipients appear to have enhanced tumor control and overall survival in GVL experiments. To better understand our in vivo findings, we utilized in vitro mixed lymphocyte reactions, where either WT or TET2-KO T-cells were co-cultured with allogeneic dendritic cells. Following allo-activation, we observed that TET2-KO T-cells proliferated significantly more than WT T-cells. Correspondingly, in cytotoxicity assays, TET2-KO T-cells exhibited higher tumor-killing activity following allo-activation in comparison to WT T-cells.

These findings provide novel insights into the pathophysiology of GVHD and GVL activity, which can ultimately lead to new therapeutic opportunities to target donor T-cells and improve HSCT outcomes.

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Deep Learning Predicts CDKN2A/B status from H&E-Stained Whole Slide Images in Peripheral Nerve Sheath Tumors

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Purpose: Loss of CDKN2A/B, a key cell cycle regulator, is a frequent early initiating event in the transformation of benign plexiform neurofibroma (PN) to pre-malignant atypical neurofibromatous neoplasm of uncertain biologic potential (ANNUBP) and ultimately malignancy. Recent guidelines proposed that biallelic CDKN2A/B inactivation is sufficient for diagnosis of ANNUBP, irrespective of histopathology. We hypothesize that deep learning models, agnostic to annotations of ANNUBP features, can learn morphologic features from hematoxylin and eosin (H&E) whole slide images (WSI) that correlate with CDKN2A/B alterations and thereby enable genotype prediction from standard histology.

Methods: We analyzed 24 peripheral nerve sheath tumors (PNSTs) (PN n = 7, atypical neurofibroma (AN)/ANNUBP n = 17) resected between 2017-2023. Tumors were molecularly characterized using TSO 500 targeted sequencing to assess CDKN2A/B status: CDKN2A/B wild type (wt) (n = 12), CDKN2A/B loss (n = 8), CDKN2A loss (n = 3), and CDKN2B loss (n = 1). Matched H&E slides were digitized and clinical histopathologic diagnoses were confirmed by an independent pathologist. From a training set of digital WSI (CDKN2A/B/AB loss: n = 9; CDKN2A/B wt: n = 9), digital image patches were extracted and encoded into feature representations using UNI, a self-supervised encoder7. These features were passed into a clustering-constrained-attention multiple-instance learning (CLAM)8 where an attention network ranked and pooled patches to generate slide-level representations. All 24 WSI, including six held-out samples, were divided into 4 validation splits, each set balanced for CDKN2A/B/AB loss (n = 3) and CDKN2A/B wt (n = 3). Attention scores were visualized as a heatmap on WSI to identify patterns predictive of mutations.

Results: The trained deep learning framework accurately predicted CDKNA2A/B status from H&E-stained WSI, achieving a mean AUC of 0.84 (Validation Split #1: 0.78; #2: 0.78,

#3: 0.78; #4: 1.0) and a mean accuracy of 0.79 (Validation Split #1: 0.83 (5/6); #2: 0.67 (4/6), #3: 0.67 (4/6); #4: 1.0 (6/6)).

Conclusions: This pilot study demonstrates that attention based deep learning models may predict diagnostically significant mutational profiles from routine H&E slides. Digital pathology-based molecular annotation has the potential to reduce reliance on sequencing, lower diagnostic costs, and improve accuracy, particularly in resource-limited settings. A significant limitation of this study, however, is the lack of an independent test dataset. Future work will therefore focus on expanding the validation dataset and conducting an independent, multi-institutional study to optimize model performance.

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Immunosuppressive 3D tumor models: pioneering combinatorial T cell therapies.

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Background: Medulloblastoma (MB) and other pediatric brain tumors have suboptimal responses to conventional therapies and remain the leading cause of cancer related death. A major roadblock to discovering more effective immunotherapies has been the shortage of preclinical tumor models that recapitulate the human tumor environment. Our team pioneered a proteogenomic pipeline to identify immunogenic peptides specific to MB. These peptides, known as tumor-specific antigens (TSA), are targeted by T cells, which then initiate a response that effectively eliminates monolayer tumor cells in vitro. However, the tumor microenvironment (TME) is immunosuppressive, due in part to resident cells derived from monocytes. To more accurately test the TSA-T cells and explore the mechanisms behind resistance to T cell therapies, we present a straightforward and replicable workflow for generating 3D in vitro spheroid cultures with infiltrating immune components.

Objectives: By providing a more representative tumor model, this method holds the promise of enhancing our understanding of the most rationale means to improve the success of T cell-based treatments.

Methods/design: To reliably replicate 3D spheres in vitro, MB cancer cell lines (D556, MB002) were cultured in specific media and conditions to enhance cell-cell interactions and replicate a cancer's stem cell (CSC)-niche. Thp1 or blood derived monocytes were added to MB 3D spheroids to replicate the immunosuppressive TME. TSA-T were manufactured and tested for cytotoxicity against immunocompetent tumor spheroids (iTS).

Results/discussion: We successfully cultured an in vitro model that begins to better represent the MB tumor's 3D architecture and microenvironment. Our data quantifies TSA-T cell cytotoxicity against MB spheroids and shows immune synapse formation with tumor cells. Using live cell analysis and confocal microscopy, we demonstrated TSA-T cell cytotoxicity against iTS and the impact of resident tumor associated monocytes. This approach better replicates tumor complexity and paves the way for more effective, personalized T cell therapies.

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Novel Agent LP-184 and Spironolactone Extend Mouse Survival in Atypical Teratoid Rhabdoid Tumor

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Atypical Teratoid Rhabdoid Tumor (AT/RT) is a pediatric brain cancer with survival rates below 40%. Illudins are a novel class of DNA-damaging alkylating agents that show efficacy against pediatric solid tumors. LP-184, a newly synthesized illudin, exhibits an enhanced ability to penetrate the brain and damage DNA in tumor cells. We hypothesized that LP-184 would work synergistically with DNA damage repair inhibitors. Spironolactone, a common diuretic with good brain penetration, can inhibit Nucleotide Excision Repair by facilitating

the ubiquitin-mediated degradation of ERCC3. ERCC3 is highly expressed in AT/RT tumors, and the combination of spironolactone and LP-184 could be a promising treatment option for AT/RT. As a single agent, LP-184 suppressed AT/RT cell lines at nanomolar concentrations (CHLA06 IC50 = 17.52 nM, BT37 IC50 = 17.94 nM, CHLA05 IC50 = 11.57 nM, CHLA266 IC50 = 44.45 nM). Immunofluorescence and Western blot assays confirmed that LP-184 reduced proliferation and induced apoptosis. Furthermore, LP-184 showed strong synergy with spironolactone. In vitro, viable cell count was significantly reduced in the combination compared to the DMSO control (BT37 = 0.0242, CHLA06 = 0.0007, CHLA05 = 0.0004). Survival studies of BT37 orthotopic xenografts in mice showed that LP-184 alone significantly reduced tumor growth and extended survival (Median survival of control = 66, Spir = 53, LP = 98, Combination = 113, p = 0.0007 by Log-rank test), though the combination with spironolactone did not result in a marked survival benefit. However, CHLA06 orthotopic xenografts in mice showed significant improvement in survival with the combination treatment, as the median survival almost tripled (Control = 27 days, combination = 75 days), and survival extension was statistically significant (p = 0.0018). Our data suggest that LP-184 is effective as a monotherapy and is further potentiated by spironolactone to reduce cell growth in vitro and extend survival in vivo. The combined treatments could provide a compelling clinical option for patients with this poor-prognosis brain tumor. Our work may inform the planned early phase clinical trials of LP-184 in children.

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Characterizing RNA Binding of Open and Closed Ezrin

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Ezrin is a member of the ezrin-radixin-moesin (ERM) family and plays a pivotal role in regulating cell morphology and cell motility. Ezrin maintains cytoskeleton dynamics and it is required for metastatic progression of osteosarcoma. Ezrin is present in the cell in two conformational states closed and open, which are also designated as inactive and active ezrin, respectively. Following phosphorylation at the threonine 567 residue (T567), ezrin acquires the open conformation. Close ezrin is localized in the cytoplasm, while the open ezrin is localized at the plasma membrane. Ezrin came up as a hit in screening experiments for RNA binding proteins. However, it is not known which state of ezrin can interact with RNA. In the closed state in the cytoplasm, it is postulated that the canonical

ligand binding sites of ezrin are inaccessible. In the current study, we investigated the RNA binding capabilities of closed and open ezrin conformations using molecular dynamics simulations. For this, we mimicked these conformations by creating T567A substitution for the closed ezrin, T567D substitution for the open ezrin and compared them to wild type protein. Herein, we first modeled and then characterized the ezrin-RNA complex via dynamic docking and 500ns of molecular dynamics simulations. The analysis of the 500ns simulation trajectory shows that there are multiple binding sites for RNA molecules for all three ezrin forms tested. We characterized the binding on each site and identified key amino acid residues in ezrin and nucleotides in RNA that were responsible for bonding interactions. We also quantified the in silico interactions by determining MM/GBSA binding free energies for each bonding. Our findings suggest that the closed/open conformational state of ezrin may have functional significance only for binding to its protein partners but not for its interaction with RNA in the cytoplasm.

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Transcriptional regulation of Neuroblastoma cellular heterogeneity by STAG1- and STAG2-cohesin

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Background: 3D genome organization defines the cell-specific transcriptional landscape through interactions between regulatory elements of target genes. In addition to its role in chromosomal segregation, cohesin complex (SMC1a, SMC3, RAD21, and STAG1/STAG2) mediates these looping structures. Neuroblastoma (NB) is highly heterogeneous with different clinical and molecular cell subtypes, marked by unique epigenetic profiles, distinct genome organization, and response to therapy. Unraveling the molecular intricacies of 3D chromatin organization in each neuroblastoma subtype holds promise for devising novel therapeutic interventions to regulate phenotypic plasticity in response to therapeutic pressure.

Aim: Detail the role and interactions of cohesin complexes (CC) in regulating the transcriptional landscape of neuroblastoma cells.

Methods: We conducted siRNA gene knockdown and utilized cell viability assays, Western blotting, and RNA-seq to study the effect of STAG1/STAG2 loss of function on NB cells.

Results: NB cells have increased expression of CC subunits compared to neural crest or adrenal gland cells (R2 database). The three core CC subunits are essential to cancer cell survival (Cancer Dependency Map) due to cohesin's role in chromosomal segregation. However, NB cells show an increased dependency on STAG2 over its paralog STAG1. STAG2 knockdown exhibited a 20% decrease in NB cell proliferation relative to the core CC and increased sensitivity to chemotherapy treatment, while STAG1 KD elicited no discernible effect. Recent studies have shown that STAG1 and STAG2-cohesin have unique and overlapping roles in gene transcription. Expression of mesenchymal transcription factors SOX9 and YAP1 increase after STAG1 loss, paired with upregulation of MES signature genes. STAG2 loss increases adrenergic transcription factors SOX4, PHOX2B, HAND2 and upregulates differentiation-related genes. These results indicate that STAG1 and STAG2 play unique roles in regulating neuroblastoma transcriptional networks.

Conclusions: Our investigation sheds light on the role of the cohesin complex, specifically STAG1/STAG2, in regulating cell-specific enhancer networks in neuroblastoma to influence differentiation and subtype plasticity.

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Elucidating the Interaction of Small-Molecule Inhibitor (S)-YK-4-279 and EWS::FLI1

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Introduction: EWS::FLI1 is a fusion oncoprotein that depends on protein-protein interactions (PPIs) with binding partners to drive Ewing sarcoma. The enantiospecific small-molecule inhibitor (S)-YK-4-279 disrupts these PPIs, and we hypothesize that it binds near alpha helix 4 of the ETS DNA binding domain (DBD) within the FLI1 portion of EWS::FLI1. We sought to determine the (S)-YK-4-279 binding site by purifying EWS::FLI1 breakpoint mutants and measuring their thermal shifts (indicating binding) via microscale thermophoresis using a Nanotemper Monolith NT.115.

Methods: Three His-tagged constructs from the FLI1 region of the EWS::FLI1 fusion protein were cloned into a pET28 expression vector. The smallest, a 16 kDa fragment, called –DBD, contains a truncation prior to alpha helix 4, while the 18 kDa TY mutant includes alpha helix 4 but is truncated 60 aa from the carboxy terminus. A 26 kDa fragment, called PY, spans from the breakpoint to the carboxy terminus. These proteins were expressed in BL21 cells induced with IPTG. An AKTA pure 25 was used to perform linear His purifications of each fragment. The fractions were analyzed by SDS-PAGE with Coomassie staining to quantify

protein concentrations, and His-Tag Western blotting to confirm the presence of the purified proteins.

Results: The SDS-PAGE and Western blot analyses confirmed the His-tagged proteins were purified to greater than 95% purity. The estimated concentrations from the preliminary purifications are: –DBD 150 µM, TY 3333 µM, and PY 77 µM. Binding data from the thermal shift is pending.

Discussion: Limitations of these purifications include high imidazole concentrations in the elution buffer. We are currently developing dialysis and concentration protocols. These data will support our efforts to characterize small molecule binding sites and could potentially be useful for future NMR studies. These protein fragments will also be used to study specific protein interactions.

Conclusion: The His-tagged –DBD, TY, and PY EWS::FLI1 breakpoint mutants can be readily purified with a straightforward linear His purification method. After dialysis and concentration, experiments using these proteins aim to identify the (S)YK-4-279 binding site and screen for other small-molecule inhibitors of EWS::FLI1.

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Dissecting the molecular mechanisms driving Lineage plasticity in High-Risk Pediatric ALL

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Background: Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, comprising 80% of pediatric cancer cases. Despite the development of risk-adapted chemotherapy and immunotherapy strategies, lineage plasticity or lineage switch (LS) has emerged as a mechanism of relapse and mortality, particularly following CD19-targeted therapies like blinatumomab and CAR-T cells, particularly in high-risk subtypes such as KMT2A-rearranged (KMT2A-r) and ZNF384-rearranged (ZNF384-r) ALL. Lineage switch involves the transition from lymphoid to myeloid phenotypes, leading to immunotherapy failure, chemoresistance, and poor survival (<12%). Thus, a deeper understanding of the molecular mechanisms driving LS is critical. This study aims to investigate the genetic and epigenetic landscape of LS in KMT2A-r and ZNF384-r ALL and identify potential therapeutic targets for post-LS disease. We hypothesize that we can uncover the cell-of-origin and molecular mechanisms of LS by developing in vitro models using KMT2A-r and ZNF384-r B-ALL cell lines and patient-derived cells to CD19-targeted immunotherapy with the

bispecific T-cell engager Blinatumomab, combined with cellular barcoding for lineage tracing.

Methods: We selected a panel of B-ALL cell lines with ZNF384 or KMT2A rearrangements, including SEM (KMT2A-AFF1), RS4;11 (KMT2A-AFF1), and KOPN-8 (KMT2A-MLLT1), and JIH-5 (EP300-ZNF384). These cell lines were characterized for lymphoid (CD19, CD22) and myeloid (CD33 and myeloperoxidase (MPO)) marker expression using flow cytometry. To establish an LS model, we co-cultured the ALL-cell lines with peripheral blood mononuclear cells (PBMCs) at a 10:1 ratio (ALL: PBMC) and treated the cells daily for 4-5 weeks with sub IC50 doses of blinatumomab (0.1, 0.5 or 1 ng/ml) that allowed for persistence of some B-ALL cells. Immunophenotypic changes were monitored weekly by flow cytometry.

Results: After 4-5 weeks of Blinatumomab treatment, a significant loss of CD19 expression was observed in ALL cell lines, indicating a potential transition away from the lymphoid phenotype. However, no significant gain in the myeloid markers CD33 or MPO was observed, suggesting that a myeloid lineage switch was not achieved in this model system. These findings suggest that while Blinatumomab induces loss of the lymphoid phenotype, a lineage switch to myeloid does not immediately occur within the current in vitro model system

Conclusion: Ongoing experiments aim to co-culture the in vitro system we developed to incorporate Bone marrow organoid models, to better mimic the in vivo microenvironment, providing insights into lineage plasticity in the context of CD19-targeted therapies and identifying new therapeutic targets for high-risk leukemia.

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Targeting the CAR T Cell DNA Methylome to Enhance Persistence and Efficacy in Pediatric Brain Tumors

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Chimeric antigen receptor (CAR) T cell therapy has revolutionized hematological cancer treatment but faces significant challenges in solid tumors due to epigenetic reprogramming and T-cell exhaustion, which limit persistence and efficacy. DNA methylation, a key epigenetic modification regulating gene expression, plays a central role in these processes. Overcoming these barriers is especially critical for pediatric brain tumors, where conventional therapies are often toxic and ineffective. This study

investigates enhancing the anti-glioma activity of engineered CAR T cells through DNA methyltransferase inhibitor (DNMTi) treatment during manufacturing. We hypothesize that targeting DNA methylation in adoptive T cells can improve immune activation and anti-tumor efficacy. We optimized a manufacturing protocol incorporating DNMTi early during the transduction of B7-H3 CAR T cells. Standard in vitro assays were conducted to evaluate the effect of various DNMTis (5-azacytadine, GSK-3484862, decitabine, and zebularine) on CAR T-cell differentiation, cytokine responses, persistence, and cytotoxicity. Results show that DNMTi treatment during manufacturing significantly improves CAR T-cell persistence and cytotoxicity. In repeated-stimulation assays, DNMTi-treated CAR T cells expanded up to 55-fold higher than untreated cells when stimulated with B7-H3-positive tumor cells. Treated CAR T cells maintained increased cytotoxicity and enhanced tumor clearance up to the 12th stimulation, with improved secretion of immune-stimulatory cytokines persisting across stimulations.Ongoing in vivo studies are assessing the persistence and efficacy of DNMTi-treated CAR T cells in xenograft and immunocompetent brain tumor models. Mechanistic studies suggest that DNMT inhibition enhances CAR T-cell activity by regulating activation and memory differentiation during manufacturing. These findings highlight the potential for DNMTi to address CAR T-cell exhaustion and improve adoptive cell therapy outcomes in brain tumors and other solid cancers.

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Mirdametinib and Paxalisib Suppress Pediatric Low-Grade Glioma Growth

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Pediatric low-grade gliomas (PLGGs) are the most common type of brain tumor in children. The most frequent activating mutations in PLGGs occur in the mitogen-activated protein kinase (MAPK) pathway, including loss of the NF1 tumor suppressor and gain of function mutations in BRAF. Downstream of these alterations, activation of the mammalian target of rapamycin (mTOR) is a hallmark of PLGG. We hypothesized that dual inhibition of MEK and mTOR could synergistically suppress the MAPK and mTOR pathways, inhibit tumor cell proliferation, and improve survival outcomes in PLGG.

To test this hypothesis, we selected mirdametinib, a novel MEK inhibitor with enhanced brain penetration that is currently in clinical trials for LGG. We also selected paxalisib, a novel mTOR/PI3K inhibitor, currently in clinical trials for diffuse midline glioma and high-grade glioma. We evaluated the synergy of MEK and mTOR inhibition in different PLGG

models harboring NF1 mutations or BRAF V600E as well as in two murine cell lines derived from Nf1−/+; Trp53−/+ cis mice.

Western blot analysis confirmed that mirdametinib effectively inhibited the MAPK pathway but triggered compensatory activation of the mTOR pathway, providing an opportunity for paxalisib to exert its synergistic effects. Additionally, the combination of mirdametinib and paxalisib significantly reduced cell proliferation, as demonstrated by BrdU staining ($P < 0.05$). Western blot analysis further revealed that combination treatment induced apoptosis in BRAF V600E-mutant PLGGs. In vivo studies supported these findings, showing that mirdametinib extended survival in the 1861 orthotopic glioma mouse model ($P = 0.001$). Furthermore, combination treatment with mirdametinib and paxalisib synergistically reduced flank tumor volume in BT40 mouse models. And Mirdametinib alone and in combination with paxalisib significantly extend the survival time of the BT40 orthotopic model. Western blot showed suppression of mTOR and MEK pathways by paxalisib and mirdametinib, respectively in murine tumors.

Mirdametinib and paxalisib synergistically inhibit tumor cell growth and induce cellular apoptosis in MAP kinase activated PLGG. Additionally, the combination treatment significantly reduces flank tumor volume in the BT40 mouse model and extends survival time of the 1861 Nf1-/- and BT40 BRAFV600E orthotopic models, highlighting its potential as a novel therapeutic strategy for PLGGs.

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Cell-free DNA shallow-pass whole genome sequencing as a non-invasive biologic assay to track non-CNS extramedullary B-ALL

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Purpose: Cell free DNA (cfDNA) has been used as a non-invasive biomarker for diagnosis, risk-adapted treatment, and disease monitoring in multiple malignancies, including adult B-cell lymphoma. Few studies, however, have used cfDNA in the context of pediatric B-cell acute lymphoblastic leukemia (B-ALL). As part of pilot study NCT05969002, we evaluate the utility of cfDNA as an adjunctive marker for disease monitoring in patients with

relapsed/refractory B-ALL enrolled on Chimeric Antigen Receptor T-cell (CART) clinical trials.

Methods: cfDNA was isolated from the plasma of 10 patients on day 0, +14, and +28 post-CART infusion. cfDNA underwent shallow whole genome sequencing (sWGS) and was assessed for genomic instability (copy number alterations (CNA), ichorCNA), fragmentomics (cfDNA fragment end motifs, non-negative matrix factorization (NMF) of fragment sizes and end motifs, genome wide short/long fragment binned ratios (DELFI)) and nucleosome footprints. We also searched each plasma sample for known CART vector sequences and VDJ clonotypes, which were determined by using Adaptive's next-generation sequencing (NGS)-based clonoSEQ® on white blood cell samples pre-CART.

Results: Plasma samples had significant genomic instability on day +14 (tumor fraction median: 0.016, interquartile range (IQR): 0.014-0.016) that did not correlate with disease burden, potentially secondary to CAR T-cell related inflammation or expansion. Fragmentomics differentiated samples with active leukemia or post-treatment from healthy controls (end-motif NMF Area Under Curve (AUC): 0.944-0.983, DELFI AUC: 0.87-0.97, size NMF AUC: 0.92-1.0) but performed modestly in distinguishing active leukemia from post-treatment states (end-motif NMF AUC: 0.501, DELFI AUC: 0.79, size NMF AUC: 0.6).

VDJ sequences were detected in peripheral blood cfDNA (2 of 14 samples), even in patients with extremely low-level disease based on clonoSEQ and flow cytometry. However, detection was not consistent, likely due to depth of coverage. CAR vector sequences were also able to be identified (6 of 20 samples).

Conclusions: cfDNA shows promise as a non-invasive adjunctive marker for disease detection and monitoring in pediatric B-ALL. Further analysis is ongoing, including ddPCR testing and integration of cfDNA with PET-CT imaging for occult extramedullary disease detection.

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Potent FGFR4-targeted antibody-drug conjugate (ADC) therapies for patients with rhabdomyosarcoma and other human cancers

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Background: Rhabdomyosarcoma (RMS) is the most common pediatric sarcoma, representing 3-4% of childhood and adolescent cancers. While multimodal therapies improved the outcomes for localized disease, 5-year survival for relapsed or metastatic RMS cases remains poor. Antibody-drug conjugates (ADCs) use specificity of monoclonal antibodies to selectively deliver potent anticancer chemotherapy agents to tumor cells while sparing healthy tissues. FGFR4, a cell-surface receptor tyrosine kinase highly expressed in RMS and other cancers such as some breast cancers, but minimally in normal tissues, is a promising immune target. We hypothesize that FGFR4-targeted ADCs could effectively treat RMS and other FGFR4-positive cancers with limited systemic toxicity.

Methods: High-affinity human FGFR4-specific binders were developed and evaluated for their internalization in RMS cell lines. The top candidate 3A11, a murine monoclonal antibody against human FGFR4, was conjugated to either monomethyl auristatin E (MMAE) via a protease-cleavable VC-PABC linker (α-FGFR4-vc-MMAE), or an exatecan derivative via a legumain-cleavable mpGlyAsnAsn(βAla) linker (α-FGFR4-mpGNN-exatecan). Efficacy of ADCs in-vitro was assessed in FGFR4-positive or FGFR4-negative cells using a live-cell analysis system. Western blots were used to validate their mechanisms of action. Finally, efficacy of these ADCs was tested in 4 subcutaneous xenograft mouse models: fusion-positive RMS (RH4), fusion-negative RMS with an FGFR4 V550L activating mutation (RMS559), a FP-RMS patient-derived xenograft (SJRHB013759), and FGFR4-positive breast cancer (MDA-MB-453).

Results: Antibody 3A11 was internalized by FGFR4-positive cells and the internalization efficiency was significantly correlated with FGFR4 surface expression levels. Both ADCs selectively killed FGFR4-expressing cells, with their potency correlating with FGFR4

expression and 3A11 internalization. Western blots confirmed that α-FGFR4-vc-MMAE ADC induced specific apoptosis and α-FGFR4-mpGNN-exatecan ADC induced cell death resulting from DNA damage in RMS cells. In 4 subcutaneous RMS or breast cancer xenograft models, both ADCs demonstrated robust antitumor activity, with α-FGFR4-mpGNN-exatecan ADC achieving superior efficacy. While α-FGFR4-vc-MMAE ADC delayed tumor growth and improved survival; α-FGFR4-mpGNN-exatecan ADC often eradicated tumors with prolonged duration.

Conclusions and Future Directions: Our results demonstrate an extraordinary efficacy of FGFR4-targeting ADCs specifically against human cancers expressing FGFR4 including aggressive rhabdomyosarcoma and breast cancers. We plan to humanize the 3A11 binder and perform pharmacokinetic and toxicology studies in non-human primates for preparation of anti-FGFR4 ADCs in clinical trials.

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Reprogramming Tumor-Associated Macrophages to Enhance CAR T Cell Therapy in Group 3 Medulloblastoma

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Background: Group 3 medulloblastoma (G3 MB) is an aggressive pediatric brain tumor (pBT) with high metastatic potential and poor prognosis. Current therapies remain inadequate and lead to significant long-term toxicities. CAR T cell therapy targeting B7-H3 offers a promising alternative, yet its efficacy is hindered by the immunosuppressive tumor microenvironment (TME) of pBTs, particularly due to tumor-associated macrophages (TAMs). Activation of TLR7/8 signaling in brain-resident macrophages has been shown to

upregulate inflammatory cytokines, potentially shifting TAMs from an immunosuppressive M2-like phenotype to a pro-inflammatory M1-macrophage state conducive to CAR T cell activity.

Objective: This study aims to optimize B7-H3 CAR T cell therapy in G3 MB by evaluating distinct B7-H3 CAR constructs and assessing the impact of TAM reprogramming through TLR7/8 activation on CAR T cell function and therapeutic efficacy.

Methods: B7-H3 CAR constructs incorporating different costimulatory domains (CD28, 4-1BB, or both) were assessed for their impact on T cell persistence and tumor cytotoxicity. Additionally, the impact of increasing concentrations of a TLR7/8 agonist, Resiquimod, formulated in poly-oxazoline nanoparticles (POx-R), on CAR T cell function and TME immunomodulation was investigated through in vitro and in vivo functional investigations, including flow cytometry-based and dPCR immuno-profiling.

Results: Structural modifications in CAR design significantly influenced anti-tumor efficacy. Preliminary data from cytotoxicity assays revealed that B7-H3 CARs with both CD28 and 4-1BB costimulation in trans outperform other CAR designs. Ongoing studies are aimed at identifying constructs with improved persistence upon repeated stimulation. Moreover, POx-R was shown to effectively penetrate the brain following systemic administration and induce repolarization of TAMs to a pro-inflammatory state characterized by increased TNF α expression, suggesting a shift to an M1 phenotype. This was supported by flow-cytometry-based immunoprofiling analyses suggesting a greater proportion of M1 macrophages in POx-R treated, G3MB-ridden mice compared to non-treated controls. While POx-R alone did not confer a survival benefit, ongoing studies are evaluating its impact on CAR T cell-mediated tumor clearance.

Conclusion: This study highlights the potential of combining optimized CAR T cell therapy with TME-targeting strategies to overcome immunosuppressive barriers in G3 MB. Further investigations are underway to determine whether TLR7/8-mediated TAM reprogramming enhances CAR T cell efficacy, with implications for improving therapeutic outcomes in pBTs.

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Evaluating in vivo efficacy of NAMPT inhibitor OT-82 in preclinical neuroblastoma models

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Previous work by our lab and others demonstrates that a subset of pediatric cancers, including Ewing sarcoma, rhabdomyosarcoma and neuroblastoma (NB), display exquisite sensitivity to inhibition of nicotinamide phosphoribosyltransferase (NAMPT). NAMPT catalyzes the rate-limiting step in one of three redundant pathways that generate nicotinamide adenine dinucleotide (NAD), an essential co-factor for energy metabolism and other downstream processes. We recently showed that molecularly diverse NB cell lines are highly sensitive to NAMPT inhibition in vitro resulting in NAD loss, DNA damage, and apoptotic cell death. Thus, we next evaluated whether these results could be recapitulated using in vivo models of NB. Three murine xenograft models of NB were injected orthotopically, monitored for growth with ultrasound and/or bioluminescence imaging (BLI), and treated with a clinical NAMPT inhibitor, OT-82, using the 3-day-on/4-day-off clinical schedule for 4 weeks. Two studies used well characterized NB cell line models, BE2C and SY5Y, monitoring with both ultrasound and BLI. SY5Y models were enrolled at a tumor volume of >50mm3; BE2C models enrolled at larger tumor volumes of >500mm3. Across both models, 15/16 vehicle mice experienced an increase in both ultrasound volume and BLI signaling, and 22/26 OT-82-treated mice experienced a decrease in both ultrasound volume and BLI signaling while on treatment, suggesting concordance between BLI and ultrasound monitoring. Among treated mice, 23/26 mice had tumor shrinkage (mean 67%; range 10%-99%). Survival to humane endpoint was 5.5 weeks and 3 weeks

longer in OT-82 treated mice compared to vehicle mice in BE2C and SY5Y models, respectively. The third xenograft study utilized a patient-derived xenograft-derived low passage cell line model and monitoring with BLI alone. A decrease in BLI intensity was observed in 10/10 mice treated with OT-82. Tumor tissue was collected at an early timepoint (3 days) for mice in both groups in all models for pharmacodynamic assessments. Tissue studies are ongoing and will be reported. Taken together, these data suggest that the efficacy of NAMPT inhibitors on NB cell lines in vitro is replicated in in vivo models, and may provide further support that NAMPT inhibitors have translational potential as a novel class of agents against NB.

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The CoREST inhibitor, corin, suppresses proliferation, increases apoptosis and promotes differentiation in malignant rhabdoid tumors

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Malignant rhabdoid tumors (MRTs) are rare and highly aggressive malignancies that primarily affect infants and young children. MRTs are characterized by an aggressive clinical course and a poor prognosis. MRTs are characterized by alterations in the SMARCB1 (INI1) gene. This gene encodes a core subunit of the SWI/SNF chromatin remodeling complex, which plays a critical role in regulating gene expression. Loss of SMARCB1 function leads to unbalanced/unopposed repression of pro-differentiation genes. CoREST is a transcriptional corepressor complex that plays a critical role in gene silencing, particularly at genes associated with differentiation. It comprises lysine specific demethylase 1 (LSD1) and histone deacetylases 1 and 2 (HDAC1/2) by the linker protein RCOR. Corin is a dual inhibitor of LSD1 and HDAC1/2 that disrupts the CoREST complex and reverses its repressive effects on differentiation genes. We hypothesized that corin would restore the ability of MRT cells to differentiate. Corin suppressed the proliferation of the MRT cell lines A204, G401, and RLM as measured by BrdU assay and Western blot for pRb. We observed increased expression of tumor suppressor proteins p16, p21, and p27. Corin induced apoptosis as evidenced by CC3 assay, Annexin V staining, and Western blot for cleaved PARP. Corin downregulated stemness markers LIN28A and LIN28B. Corin increased the expression of the neuronal marker NCAM-L1. Tumors treated with corin showed increased bone and skeletal muscle differentiation histologically, suggesting that corin may promote differentiation in MRTs. Corin treatment led to a significant reduction in flank tumor size compared to the control group, further supporting its therapeutic

potential. Corin shows promise as a therapy for MRTs by reducing proliferation, inducing apoptosis, decreasing stemness, and promoting differentiation, ultimately leading to significant tumor reduction in vivo.

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Defining Phenotypic and Functional Differences of Activated NK Cells to Improve Cancer Immunotherapy

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Background: Natural Killer (NK) cells have powerful anti-tumor effect as innate lymphoid cells. Infusion of healthy donor expanded NK cells shows promise for treating acute myeloid leukemia (AML). NK cells expanded for clinical trials are commonly co-cultured with irradiated K562 “feeder” cells expressing membrane bound IL-15 or IL-21 and 41BB ligand plus supplemental cytokines to sustain an activated phenotype. Our goal is to define the unique surface protein expression, transcriptional phenotype, and methylation patterns of each NK cell expansion method.

Methods: To expand human NK cells, CD3+ cells were depleted from human donor peripheral blood mononuclear cells. Remaining cells were co-cultured for 10 days with: (1) K562.mbIL15.41BBL + IL-2; (2) K562.mbIL21.41BBL + IL-2; (3) K562.mbIL21.41BBL + IL-2 + TGFβ. We created a multiparameter flow panel including activating and inhibitory NK cell receptors. Genomic DNA and RNA were isolated from expanded and non-activated NK cells. Whole genome bisulfite sequencing (WGBS) reported methylated regions, which were detected using BSmooth and InformME packages. RNA sequencing was analyzed with DESeq.

Results: There were no differences in NK cell purity or expansion between the cohorts. We found distinguishing NK cell subsets amongst our expanded NK cells based on activation, inhibitory and exhaustion receptors. Our expanded NK cell products have unique upregulation in transcriptional pathways: (1) MAPK and mTOR signaling, TNFα signaling via NF-KB; (2) INFα/γ response; (3) IL7, IL9 and IL10 signaling. Mean methylation levels of each group significantly differed with hypomethylation of (1) RUNX1; (2) cell cycle; (3) p53 pathway and hypermethylation of (1) NOTCH and wnt signaling; (2) SMAD2/3 and IL7 pathway; (3) TCA cycle.

Conclusions: Through comprehensive evaluation of transcriptomic, epigenomic, and surface protein signatures, we have discovered defining NK cell features post-activation.

We plan to test functionality of activated NK cells to relate choice of ex vivo expansion methodology to ideal immunotherapeutic product.