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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the first year of this project, we achieved significant progress in deciphering the complex epigenetic landscape governing Diffuse Intrinsic Pontine Gliomas (DIPG). The primary aim was to understand the relative contributions of K27 methylation and S31 phosphorylation to gliomagenesis in H3.3 K27M mutant DIPG. We focused on the role of histone modifications, specifically mutations in histone H3.3, to discern their impact on cellular functions like chromosome segregation and gene expression. Utilizing CRISPR-Cas9 technology, we successfully generated and characterized knockout variants for EZH2 and EZH1, histone methyltransferases responsible for H3K27 methylation. The cells also harbor mutations in H3.3 at K27 and S31 sites, laying the groundwork for multifaceted functional analyses.
Preliminary data strongly validate our hypothesis that chromosome missegregation and epigenetic dysregulation contribute to tumorigenesis. We observed that K27M mutations induce chromosome

15. SUBJECT TERMS

NONE LISTED

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Defining the relative contribution of K27 methylation and S31 phosphorylation to gliomagenesis in DIPG

Year 1 Progress report

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1. Introduction

Diffuse intrinsic pontine gliomas (DIPG) are aggressive high-grade gliomas of the brainstem. They are very rare cancers and almost always occur in the pediatric population, with approximately 200 to 300 new cases annually in the United States[1]. With a median overall survival of only 9–15 months, they are among the most lethal of all human cancers. Standard of care is limited to surgical resection and radiotherapy [2].To date, no clinical trial of a chemotherapeutic agent has shown any survival benefit for DIPG[2]. The **outcome** of this work will be the identification of the mechanism behind DIPG, the discovery of therapeutic targets and development, and validation of a novel tumor-specific model that can also be used to support clinical trials. Thus, this work address two FY21 RCRP Focus Areas: Biology and Etiology: Identify disease-defining molecular pathways and Research Model: Develop and validate rare tumor-specific models that can support clinical trial readiness.

Heterozygous mutations in histone variant H3.3 (H3F3A) occur as early events in DIPG (prior to p53 mutations)[3,4] and are sufficient to drive a global reduction of Lys 27 (H3 K27) bi- and tri- methylation (me2, me3) on all histone H3 variants. Histone H3 K27 methylation is responsible for epigenetic gene repression and loss of K27 Me3 is associated with epigenetic dysregulation in $H3.3^{K27M}$ cells[2,5–9]. The preeminent model for H3.3 mutations in DIPG is that $H3.3^{K27M}$ mutants drive tumors because of a loss of epigenetic gene repression[10–13].

Our **hypothesis** is that chromosome missegregation and epigenetic dysregulation contribute to tumorigenesis in H3.3 tumors (**Fig. 1**). To address our hypothesis we will prevent K27 methylation without using K27M dominant negative mutations as they reduce S31P in mitosis and promote chromosomal instability (CIN) by using genetic knockouts of the histone methyltransferases, EZH1 and EZH2 that catalyzes H3K27 methylation [14,15], in tumors and cell lines, and assess the effect on K27M and S31 mutant and wildtype tumor cells.

Emerging data strongly supports our hypothesis. In preliminary data we find that the K27M mutations induce chromosome missegregation and aneuploidy. Histone H3.3 has a unique Chk1 phosphorylation site, Ser31 (S31) that is critical for mitotic checkpoint regulation[16]. Chromosome missegregation in non-transformed, diploid cells activates a p53-dependant G_1 cell cycle arrest, which blocks the proliferation of normal cells that inadvertently become aneuploid[17]. Masking of S31 phosphorylation (S31P) results in abrogation of cell cycle checkpoints that monitor the alignment and segregation of chromosomes [16]. We find that that H3.3 K27M is defective for S31P *in vitro*. DIPG cell lines have decreased levels of mitotic S31P, and are chromosomally unstable and aneuploid [2]. Genomic-editing of H3.3 to revert the K27M mutation restores mitotic S31P (and K27Me3), and significantly decreases the rate of chromosome instability. Expression of H3.3 K27M or a non-phosphorylatable S31A mutant in normal, diploid cells and astrocytes results in chromosome missegregation and cell cycle checkpoint defects, and cells fail to undergo G_1 cycle arrest in response to missegregation, leading to aneuploidy. This K27M effect is suppressed by co-expressing a phospho-mimetic S31E that restores S31P (K27M/S31E). Expression of H3.3 K27M or S31A inhibits p53 accumulation. The overall **goals** of this application is to define the **relative contributions** of loss of K27 methylation and S31 phosphorylation to gliomagenesis in H3.3 K27M mutant DIPG.

2. Keywords

Keywords DIPG, H3.3 K27 Methylation, S31 Phosphorylation, EZH2, EZH1, Tumorigenesis

NOTE DIPG is now known as DMG, we are still using DIPG here for consistency with the SOW.

3. ACCOMPLISHMENTS:

What were the major goals of the project? The overall **goals** of this application is to define the **relative contributions** of loss of K27 methylation and S31 phosphorylation to gliomagenesis in H3.3 K27M mutant DIPG. To this end we developed a novel mouse model. In this mouse model DIPG gliomas develop from neural stems cells following expression of PDGFB linked to H3.3, PDGFB - $H3.3^{K27M}$ and $H3.3^{S31A}$ promoted the development of high-grade gliomas whereas $H3.3^{WT}$ controls did not. As $H3.3^{S31A}$ is WT for K27 methylation (IF, WB, IHC and MS/MS), our data demonstrates that loss of S31P is oncogenic. It is unclear if H3.3 mutations contribute to tumorigenesis via disruption of pericentromeric heterochromatin and chromosome missegregation via suppression of S31P or alterations in the epigenetic landscape via loss of lysine methylation or mostly likely via both.

Specific Aim 1: Determine the effect of H3.3 K27 lysine methylation on growth, chromosomal instability, gene expression and S31 phosphorylation in DIPG. We will create EZH2 and EZH1 knockout variants in H3.3 K27M, H3.3 WT, S31E, and S31A mutant DIPG cell lines using CRISPR-Cas9. We will analyze cells expressing H3.3, EZH1 and EZH2 mutations by live-cell imaging, MS/MS, and RNA-Seq. We will define the consequences on post-translational histone modifications, gene expression, and chromosomal instability

Specific Aim 2: Determine the contribution of loss of H3.3 K27 lysine methylation and serine 31 phosphorylation to tumorigenesis in DIPG. We will establish if there is cooperation or rescue in the overall survival or tumor grade using our novel mouse model in the context of EZH2 deletion and loss of K27 methylation in H3.3 K27M, H3.3 WT, S31E or S31A P2A-PDGF driven tumors.

Our project's major goals, as outlined in the approved **Statement of Work (SOW**), encompassed the following key objectives: To investigate the impact of genetic modifications, specifically CRISPR-Cas9 knockout of EZH2 and EZH1, as well as the introduction of H3.3 K27M and S31A mutations. To establish a reliable DIPG mouse model for studying epigenetic changes in gliomagenesis. To validate the efficacy of these genetic modifications through comprehensive experimental approaches.

Milestones Completed: Year 1

Specific Aim 1: Effect of H3.3 K27 Methylation in DIPG
Task 1.1: CRISPR-Cas9 creation of EZH2 and EZH1 knockout variants: Completed
Subtask 1.1.1: H3.3 K27M variants: Completed
Subtask 1.1.2: H3.3 WT, S31E, and S31A variants: Completed
Task 1.2: Analysis through live-cell imaging, MS/MS, and RNA-Seq: In Progress
Subtask 1.2.1: Live-cell imaging: Ongoing
Subtask 1.2.2: MS/MS: In Progress
Subtask 1.2.3: RNA-Seq: Started (we have results for some cell line pairs)

Specific Aim 2: Contribution to Tumorigenesis in DIPG
Task 2.1 Establishment of mouse model: Completed
Task 2.2: Evaluation of overall survival or tumor grade: In Progress
Subtask 2.2.1: Context of EZH2 deletion: In Progress (mice crossing)
Subtask 2.2.2: Loss of K27 methylation: Not Started

What was accomplished under these goals?

During this reporting period, significant progress has been made in achieving these major goals:

A. Comprehensive CRISPR-Cas9 Manipulation of EZH2, EZH1, and H3.3: We have successfully executed CRISPR-Cas9 knockout techniques to target EZH2 and EZH1 genes, as well as introduced H3.3 K27M and S31A mutations in the H3.3 gene. The knockout and mutation procedures are critical for understanding the role of these genes and their respective proteins in Diffuse Intrinsic Pontine Glioma (DIPG). We now have 5 paired cell lines that encompass single knockouts of EZH2 and EZH1, double knockouts, and controls (SF8628, SF7761, SF9427 DIPG cells and two adult GMB cell lines U118. We have also incorporated H3.3 WT and H3.3 K27M mutations in each line using CRISPR-Cas9. The diverse set of cell lines enhances the breadth and depth of our study, enabling complex investigations into epigenetic deregulation in DIPG. This cellular toolkit sets a

robust platform for both current and future research into DIPG and cancer epigenetics. The genetic modifications have set the stage for a wide array of functional and mechanistic studies, allowing us to delve deeper into epigenetic regulation and its impact on DIPG pathology. Planned for Year 2 including live cell imaging of chromosomes missegregation and gene expression analysis using RNAseq. Verification and Validation: Employed Western Blotting, DNA Sequencing, and Immunofluorescence to confirm gene knockouts and mutations, ensuring the efficacy of our experimental approach. We also noted H3.3 K27M
leads to loss of K27me and S31A.

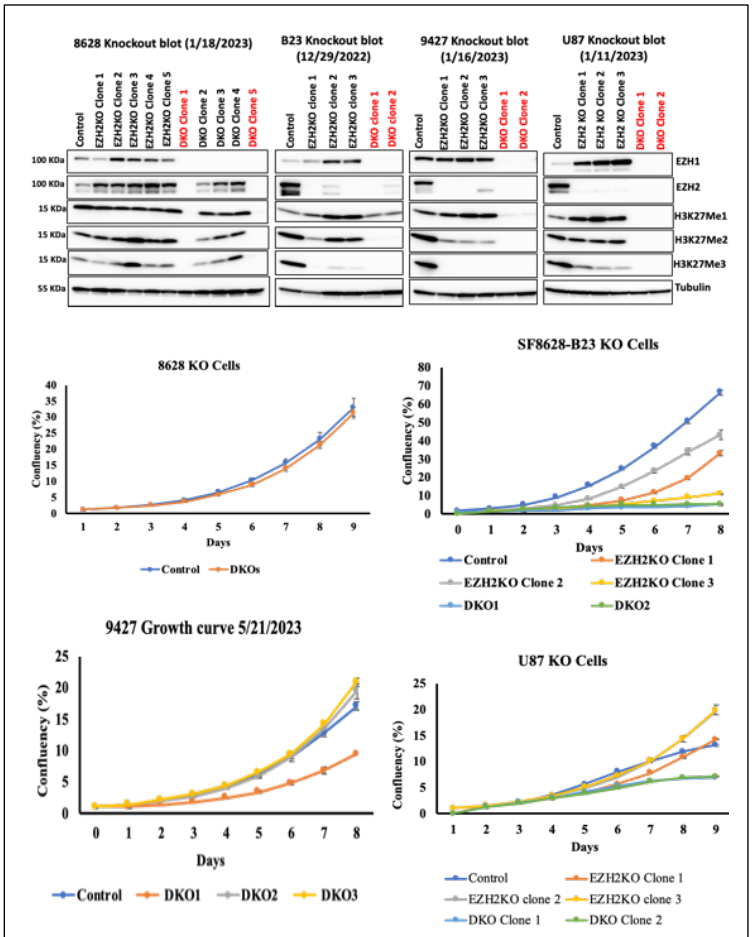


Figure 1: CRISPR-Mediated EZH1 and EZH2 Knockout in DMG and GBM Cells. Two guide RNAs were designed to target EZH1 (gRNA 1: TTGGTAGTTGTACACTTGTG; gRNA 2: GGATTGGGATCTTTGCCAAG) and EZH2 (gRNA 1: TAGCAAAGATGCCTATCCTG; gRNA 2: CAGGATGAAGCAGACAGAAG). These guide RNAs were cloned into the Lenti-Cas9-GFP plasmid (124770, Addgene) and DMG and GBM cells were transfected (not used as a virus). Subsequently, the cells were allowed to recover for 3 days, and GFP-positive cells were sorted using flow cytometry. Single-cell selection was conducted, and two independent clones were chosen and expanded. Live-cell proliferation curves were generated using an Incucyte, showing the growth of SF8628 EZH1/2KO, SF8628-B23 EZH1/EZH2KO, SF9427 EZH1/2KO, U87 EZH1/2KO, U118 EZH1/2KO, along with their respective parental cells. In each well of a 24-well plate, 20,000 cells were seeded and allowed to proliferate for 8 days, and their growth was compared.

Clines highlighted in red used for DKO experiments.

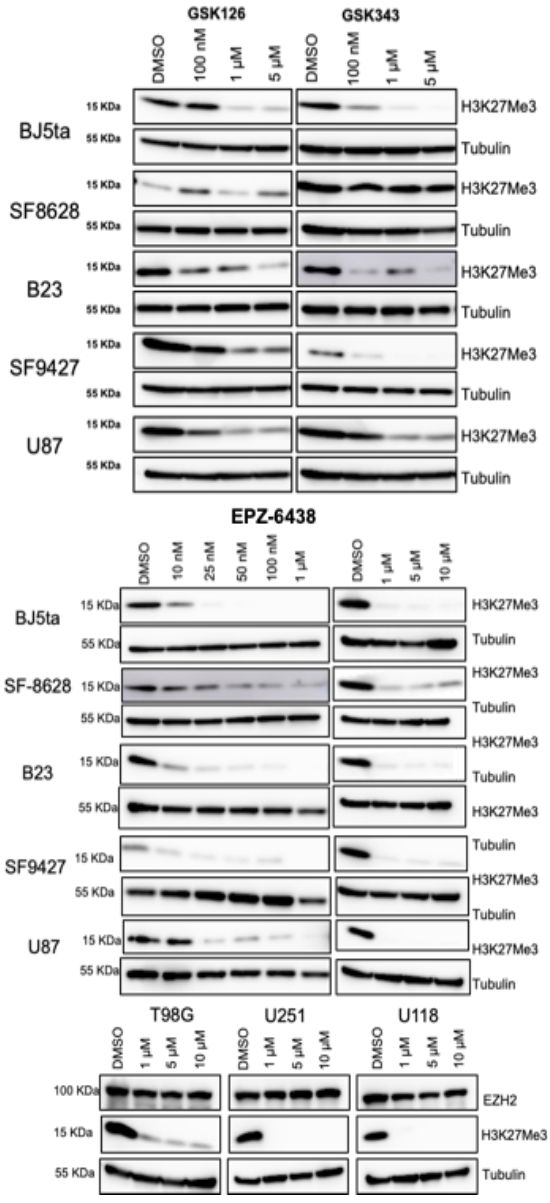
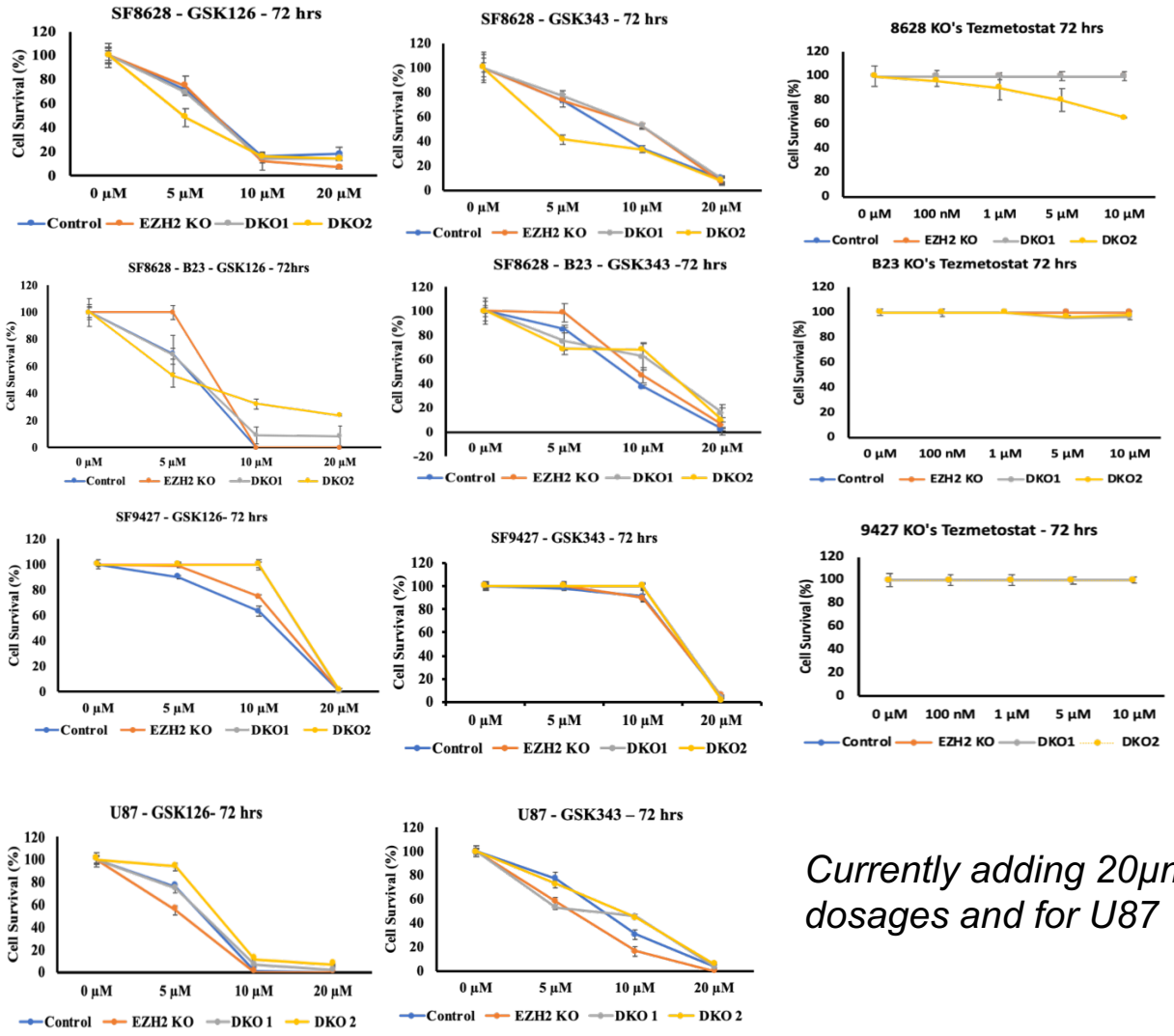


Figure 2: Effects of GSK126, GSK343, and EPZ-6438 on H3K27Me3 Levels. Inhibition of H3K27Me3 - DMG and GBM cells were treated with 100 nM, 1 μM, and 5 μM of GSK126 and GSK343, whereas EPZ-6438 was used at 10 nM, 25 nM, 50 nM, 100 nM, and 1 μM for 72 hrs. Cell lysates were prepared, and H3K27Me3 expression was determined by western blot.



Currently adding 20µm dosages and for U87

Figure 3B

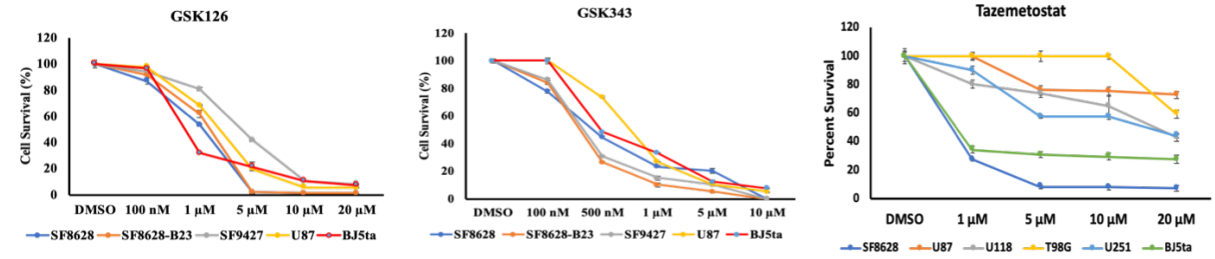


Figure 3: Response of Parental and EZH1/2 Knockout Cells to EZH2 Inhibition (72-Hour MTS Assay), Cell Growth and Survival Assay (21 Days), and Impact of Drug Treatments. Figure 3A Response of Parental and EZH1/2 Knockout Cells to EZH2 Inhibition (72-Hour MTS Assay). Cells, including parental and EZH1/2 knockout cells, were treated with different inhibitors, including GSK126, GSK343, and *EPZ-6438 (*Tazemetostat), for 72 hours. The use of knockout cells allowed us to pinpoint that only Tazemetostat prevented growth due to EZH2 and EZH1 inhibition, while the other drugs resulted in cell death and inhibited growth due to off-target toxicity.Figure 3B: Cell Growth and Survival Assay (21 Days). Cells were treated with EZP-6438 at various concentrations for two weeks. The colonies were stained with crystal violet, and absorbance at 450 nm was measured to determine cell survival. These experiments provide critical insights into the differential effects of various inhibitors on cell growth and survival, shedding light on the potential for Tazemetostat as a targeted therapy and emphasizing the importance of minimizing off-target toxicity in drug development.

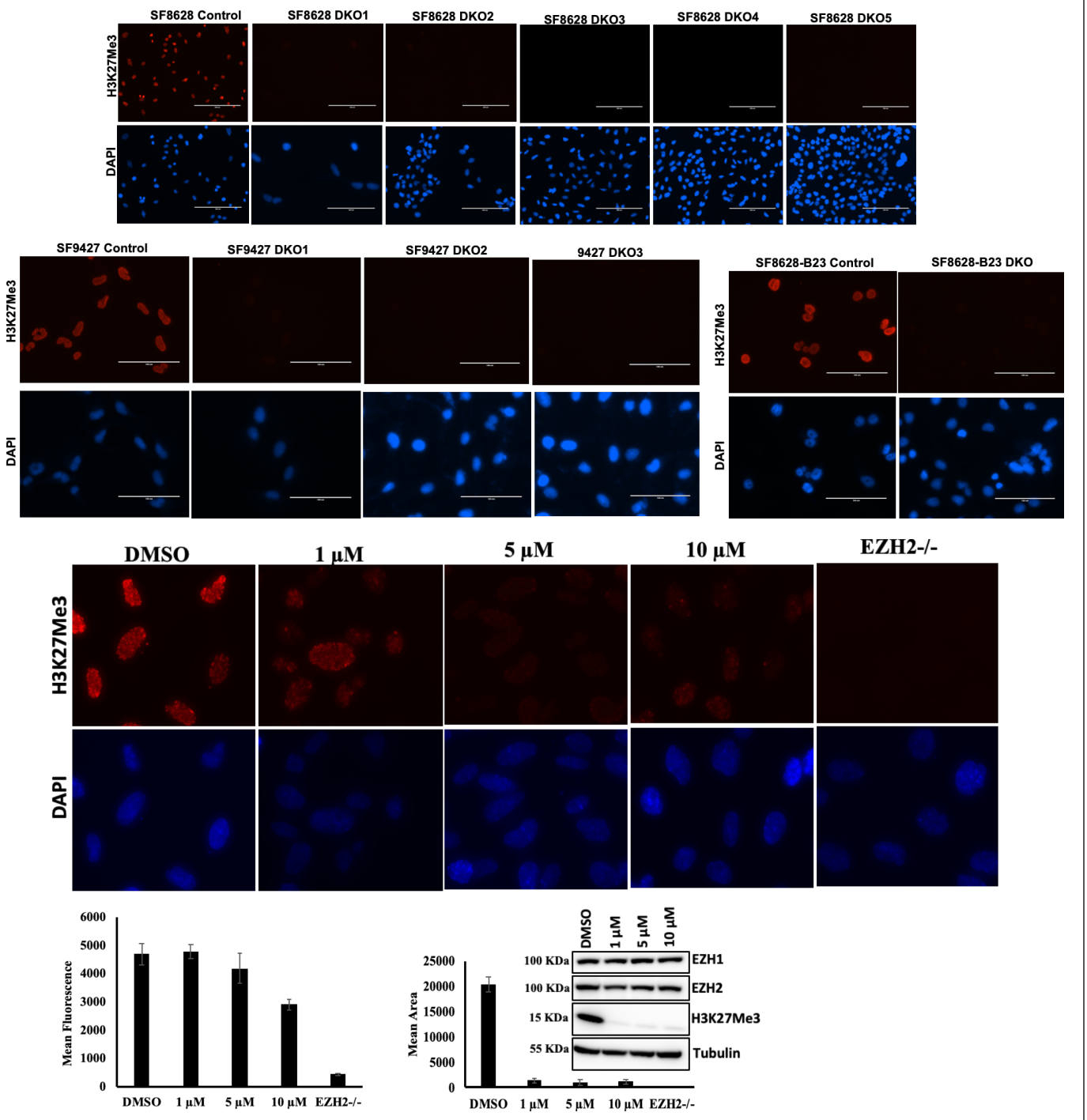


Figure 4. Immunofluorescence Analysis of H3K27Me3 in EZH1 and EZH2 Knockout DMG and GBM Cells. Selective clones shown for brevity. Comparative Analysis of H3K27me3 Levels in Response to Tazemetostat Treatment and EZH2 & EZH1 knockout. This figure illustrates a comparative analysis of H3K27me3 levels in SF9247 cells treated with Tazemetostat. Two distinct assessment methods, Western blotting and immunofluorescence, were employed to evaluate the impact of the treatment on this crucial epigenetic modification. Notably, a complete loss of K27me3 was observed exclusively in the EZH1 and EZH2 knockout cells, highlighting the significance of these histone methyltransferases in maintaining H3K27me3 levels.

B. DIPG Mouse Model: Successfully engineered an RCAS/TVA mouse model that faithfully recapitulates the characteristics and progression of Diffuse Intrinsic Pontine Glioma (DIPG). Expression of H3.3 K27M or a non-phosphorylatable S31A mutant in normal, diploid cells and astrocytes results in chromosome missegregation and cell cycle checkpoint defects, and cells fail to undergo G_1 cycle arrest in response to missegregation, leading to aneuploidy. This K27M effect is suppressed by co-expressing a phospho-mimetic S31E that restores S31P (K27M/S31E). Expression of H3.3 K27M or S31A inhibits p53 accumulation. We are investigating if the use of EZHIP expression rather than EZH2 deletion would. This validated DIPG mouse model has become a shared resource within the research community, accelerating collaborative efforts to unravel the complex biology of DIPG and test innovative therapies. Future Applications: The DIPG mouse model is poised to facilitate high-throughput drug screening and gene therapy experiments, thus catalyzing breakthroughs in both basic and translational DIPG research. Our mouse model provides a critical tool that brings us a significant step closer to understanding the underpinnings of DIPG and developing effective treatments.

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

Opportunities for training and professional development have been provided to project team members and collaborators. These activities have enhanced their proficiency and knowledge in the field, contributing to their professional growth. This has included specific training in advanced live cell imaging by expert and CO-PI Hinchcliffe. These included attending the 2022 Society for Neuro-Oncology Annual Meeting in Tampa with lab personnel to present data and learn form leaders in the field.

How were the results disseminated to communities of interest?

Results of our research have been actively disseminated to communities of interest through various means, including peer-reviewed paper preparations, presentations at conferences such as the Annual Meeting of the Society of Neuro Oncology (2022), and outreach activities targeting diverse audiences.

Our efforts aim to enhance public understanding and foster interest in science, technology, and the humanities.
A Peer-reviewed paper on growth and survival CRISPR knockouts of EZH1/2 in revision / preparation as were required to added the testing of three EZH2 inhibitors after reviewers requested this control. Presentation at the Annual Meeting of the Society of Neuro Oncology (2022)

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

In the upcoming reporting period, we plan to continue our research endeavors to accomplish the project's goals and objectives as outlined in the approved SOW. This includes completing pending analyses, initiating new experiments, and preparing for the dissemination of findings. Our commitment to advancing scientific knowledge and addressing the challenges posed by DIPG remains steadfast.

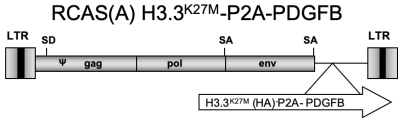


Fig 5. Schematic of RCAS viral vector providing linked PDGFB and H3.3 delivery and expression.

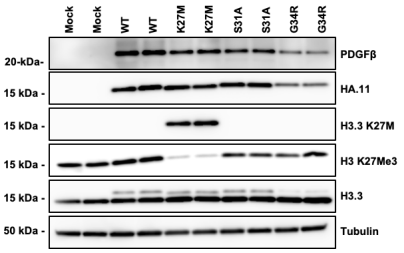


Fig 6. Expression of H3.3 K27M mutants causes a global reduction of K27 Me3. Nestin TVA+ astrocytes infected with RCAS H3.3 -P2A-PDGFβ, $H3.3^{K27M,}$ $H3.3^{S31A}$ or $H3.3^{WT}$

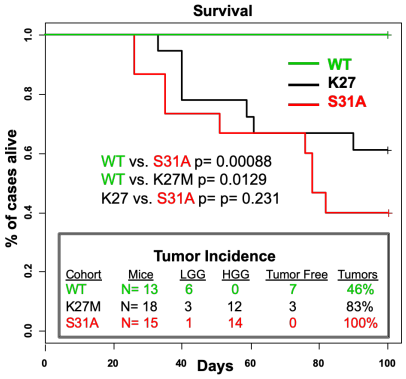


Fig 7. . Survival and tumor incidence in a N-TVA mice. N-TVA mice were infected with RCAS(A) $H3.3^{K27M}$-P2A-PDGFβ, **$H3.3^{S31A}$**-P2A-PDGFβ or $H3.3^{WT}$-P2A-PDGFβ.

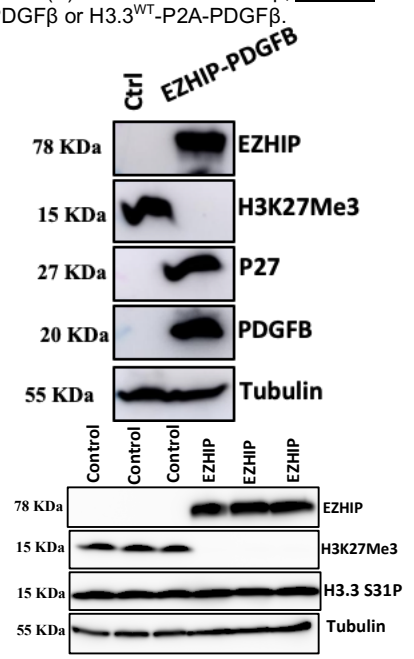


Fig 8. Expression of RCAS-EZHIP causes a global reduction of K27 Me3. Nestin TVA+ astrocytes infected using DF1s expressing RCAS EZHIP- -P2A-PDGFβ.- Mice with this new virus and they are now getting brain tumors.

Tasks Planned for Years 2-3

Year 2
Task 1 (Calendar Months 1-3):
Complete pending MS/MS analyses for Specific Aim 1.
Initiate RNA-Seq for Specific Aim 1.

Task 2 (Calendar Months 4-6):
- Finalize RNA-Seq analyses for Specific Aim 1.
- Update mouse models for Specific Aim 2.

Task 3 (Calendar Months 7-9)
Initiate long-term effects analysis in mouse models.
Evaluate overall survival or tumor grade in EZH2 deletion context.

Task 4 (Calendar Months 10-12)
Complete long-term effects analysis.
Prepare for dissemination of Year 2 findings.

4. IMPACT

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

Our research has significantly improved our understanding of DIPG and how it works. Think of DIPG as a puzzle, and we've found some crucial pieces. We focused on two specific things, H3.3 K27 methylation and S31 phosphorylation, which are like special clues in this puzzle. Our discoveries have not only helped us understand DIPG better but have also opened up new possibilities for treating it. Imagine we've found a new path to explore in the forest of DIPG, and this path might lead us to a way to fight this tough disease. However, it's important to know that the first year was mostly about preparing the tools we need for this journey. Our goal is to find better treatments for DIPG, especially for kids who have it. So, while our work might seem complex, it's all about making a difference in the lives of people affected by this disease. We have a great team with different skills, like detectives on a mission, and we're determined to find solutions to help those who need it most.

What was the impact on other disciplines?
Our work extends its influence beyond the boundaries of epigenetics and cancer research. The findings, methodologies, and models developed in this project hold promise for impacting fields such as neurology and oncology. Moreover, the interdisciplinary nature of our research encourages collaboration between different scientific disciplines, fostering innovative approaches to complex problems.

What was the impact on technology transfer?
While it's still early in our project, we see exciting potential for our work to reach beyond the boundaries of our own field. Imagine our project as a seed that has just been planted, and we're waiting to see it grow. Right now, our focus is on creating special tools in the lab to use in year 2. These tools are like the building blocks of future discoveries. In the future, we will share those cell tools and , we hope our research will not only benefit the field of cancer research but also lead to new ways of diagnosing and treating diseases.

What was the impact on society beyond science and technology?
Our project is still in its early stages, but we believe that its potential impact reaches far beyond the world of science and technology. It's like a small spark that has the potential to ignite significant changes in society.
Our work is focused on understanding a rare and challenging disease, DIPG, which mostly affects children. By shedding light on the mysteries of this illness, we hope to improve not only our scientific knowledge but also the lives of those affected. Imagine a future where parents of children with DIPG have more hope because of our research. Think about how our discoveries could lead to better treatments, better policies, and more support for families facing this devastating disease. Our project has the power to change lives and bring about a brighter future for those touched by DIPG, and that impact extends far beyond the laboratory and into the hearts of people in our society.

In summary, our project has achieved significant milestones and progress in advancing the understanding of DIPG and its implications for the broader scientific community and society as a whole. We remain committed to our goals and objectives as outlined in the approved Statement of Work (SOW) and look forward to continuing our impactful research.

5. **CHANGES/PROBLEMS:**

   a. **Changes in approach and reasons for change**

      We were prepared to publish our initial assessment of the knockout clones before conducting RNA-Seq and mass spectrometry analyses to specify those findings. However, we were advised to contrast our PRC2 CRISPR mutants with all commonly used drugs to demonstrate the superiority of the genetic approach. We intend to publish to demonstrate productivity and to simplify communication in the expression and proteomic analysis manuscripts to follow. We believe that these reviewer requests have improved this project.

   b. **Actual or anticipated problems or delays and actions or plans to resolve them**

      We had hoped to start live cell imaging of the cells for chromosomal missegregation, but validating the clones has taken longer than expected. Additionally, the microscope was briefly out of action due to a new operating system installation. During this delay, we utilized the time to perform the requested drug experiments.

   c. **Changes that had a significant impact on expenditures**

      Inflation is hitting us hard- all price are up.

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

      NA

   e. **Significant changes in use or care of human subjects NA**

   f. **Significant changes in use or care of vertebrate animals. NA**

   g. **Significant changes in use of biohazards and/or select agents NA**

6. **PRODUCTS**

Publications, conference papers, and presentations

Journal Publications: We have manuscripts nearly ready for peer-reviewed journals for publication. Any publications will acknowledge federal support.

Other Publications: Conference Papers, and Presentations*: We presented our research findings at the Annual Meeting of the Society of Neuro Oncology in 2022. This presentation, titled "Exploring the Molecular Mechanisms of DIPG," generated significant interest and discussions within the research community. Additionally, our team is in the process of preparing conference papers based on our research outcomes. One of these papers will be submitted to the International Symposium on Pediatric Neuro-Oncology (ISPNO) in 2023.

See appendix for a full list of our related abstract.

Website(s) or other Internet site(s). NA due to our animal research we do not make specific mentions of products on our institutional web sites.

Technologies or Techniques: Our research has resulted in the development of novel cell lines with H3.3 K27M and S31A mutations, representing valuable tools for studying DIPG and epigenetic dysregulation. These cell lines will be made available to the scientific community to advance research in this field. Detailed protocols for using these cell lines are provided on our project website.

Inventions, Patent Applications, and/or Licenses: While our project has not generated patentable inventions during this reporting period, we are actively exploring the possibility of patent applications for any novel technologies or discoveries that may arise in the future phases of our research.

Other Products: In addition to the above outcomes, our project has led to the establishment of a comprehensive biospecimen collection, including CRISPR DIPG cell lines and matched parental cells. These biospecimens are stored under standardized conditions and will be made available for collaborative research projects. Our project remains committed to sharing our findings, resources, and tools with the scientific community to advance our understanding of DIPG and improve outcomes for those affected by this devastating disease.

PRODUCTS:

1. Peer-reviewed paper on growth and survival CRISPR knockouts of EZH1/2 in preparation

We added the testing of three EZH2 inhibitors after reviewers requested

2. Presentation at the Annual Meeting of the Society of Neuro Oncology (2022)

Year 1 milestones substantiate progress toward understanding the roles of H3.3 K27 lysine methylation and serine 31 phosphorylation in DIPG. Future work is planned to deepen these insights. We should be able to address our overall hypothesis that chromosome missegregation and epigenetic dysregulation contribute to tumorigenesis in H3.3 tumors in year 2 by conducting the live cell imaging and RNAseq experiments.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	James Robinson
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.8 person months
Contribution to Project:	
Funding Support:	Melanoma Research Alliance

Name:	Edward Hinchcliffe
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.36 person months
Contribution to Project:	
Funding Support:	

Name:	Lukmanul Hakkim Faruck
Project Role:	Researcher
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4 person months
Contribution to Project:	
Funding Support:	

8. APPENDICES:

References

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B. Abstracts

Abstract- Hormel Institute symposium, August 2-3, 2023

Gene (On/Off) target validation of EZH2 inhibitor in diffuse midline glioma

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Diffuse midline glioma (DMG) is a particularly aggressive form of pediatric brain cancer, with a median survival time of a mere 15 months. The most common mutation identified in DMG is the somatic Histone 3 lysine 27 to methionine mutation (H3K27M), resulting in a broad decrease in H3K27 trimethylation (H3K27Me3) and subsequent epigenetic imbalance. The EZH2 protein, a component of the polycomb repressive complex 2 (PRC2), initiates H3K27Me3, thus enforcing gene silencing. Within DMG, the mutated H3K27M peptide impairs EZH2's catalytic function, leading to a reduction in H3K27Me3. However, the wild-type H3K27 peptide is still subject to methylation by EZH2, resulting in persistent H3K27Me3 on select genes involved in differentiation and cell cycle control, thus fostering DMG's malignant and undifferentiated traits. Targeting the residual H3K27Me3 presents a promising therapeutic approach, yet most targeted inhibitors have proven ineffective in clinical trials due to off-target consequences and resultant toxicity. The EZH2 inhibitors GSK126 and GSK343 were discontinued in clinical trials due to their toxic side effects. Meanwhile, EPZ-6438 was approved by the FDA for follicular lymphoma, but with a warning regarding potential secondary cancers. To repurpose these EZH2 inhibitors for DMG treatment, validating their genetic targets in DMG models is a critical step. Responding to this need, we employed the CRISPR/Cas9 method to establish EZH2 knockout DMG cells, and then scrutinized the target specificity of GSK126, GSK343, and EPZ-6438. Our investigations revealed that GSK126 and GSK343 both instigate cell death in EZH2 knockout DMG cells and non-transformed human cells, demonstrating adverse off-target effects. Conversely, EPZ-6438 does not provoke cell death in EZH2 knockout DMG cells. Nonetheless, while

EPZ-6438 moderately impedes the growth of H3K27M DMG cells, it fails to entirely suppress H3K27me3. Recent discoveries have emphasized the overexpression of CXorf6, which encodes the EZH2 inhibitor protein (EZHIP), in posterior fossa ependymomas (PFE) and H3K27M negative DMGs. EZHIP interacts with the catalytic site of EZH2 in a manner akin to the H3K27M, leading to suppression of H3K27me3. Retaining residual H3K27Me3 on certain genes manifest PFE. We aim to elucidate EZHIP's role in tumorigenesis and characterize the structure of the EZHIP-PRC2 interaction, to facilitate the development of novel PRC2 inhibitors for pediatric glioma treatment.

Abstract accepted – AACR SPECIAL CONFERENCE IN CANCER RESEARCH: BRAIN CANCER. October 19-22, 2023, Hilton Minneapolis, Minneapolis, Minnesota

EZH2 as a therapeutic target for glioma.

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Diffuse midline glioma (DMG), a highly aggressive pediatric brain cancer, and glioblastoma multiforme (GBM), an intractable disease, pose significant clinical challenges. DMG is characterized by a dishearteningly short median survival period of 15 months, and GBM by a low five-year survival rate of merely 6.9 percent. A critical player in these malignancies is Enhancer of zeste homologue 2 (EZH2), a subunit of the polycomb repressive complex 2 (PRC2) that catalyzes histone 3 Lysine 27 tri-methylation (H3K27Me3). This modification suppresses gene expression, orchestrating cell fate. Dysregulated EZH2, linked to both solid and hematological malignancies, including brain cancers, heralds a poor prognosis. DMG presents a heterozygous H3K27M mutation that cripples EZH2 functionality, causing epigenetic dysregulation via H3K27Me3 loss. However, intriguingly, DMG retains residual H3K27Me3 on certain genes, propelling gliomagenesis. EZH2 overexpression in GBM and persisting H3K27Me3 in DMG underscore the malignancy. Therefore, EZH2 has emerged as a potential therapeutic target for gliomas. Despite this promise, targeted inhibitors often fail in clinical trials, thwarted by off-target effects and consequential toxicity. EZH2 inhibitors GSK126 and GSK343 were halted in trials due to their harmful side effects. Meanwhile, EPZ-6438, a more selective EZH2 inhibitor, received FDA approval for treating follicular lymphoma and advanced epithelioid sarcoma. However, recent studies found survival prolongation but subsequent recurrence and resistance in a pediatric brain tumor PDX model with high EZH2 expression treated with EPZ-6438. Recurred tumor cells exhibited minimal EZH2 expression and reduced H3K27Me3 levels, suggesting EPZ-6438 resistance. To delve into these findings, we utilized CRISPR/Cas9 technology to establish EZH2 knockout DMG and GBM cells, and subsequently examined the target specificity and anti-cancer potential of GSK126, GSK343, and EPZ-6438. Our investigations unveiled that both GSK126 and GSK343 induced cell death in EZH2 knockout DMG and GBM cells and non-transformed human cells, indicating detrimental off-target effects. In contrast, EPZ-6438 did not trigger cell death in EZH2 knockout cells but only moderately inhibited the growth of DMG and GBM cells, but also failed to fully suppress H3K27Me3. By establishing EZH2 knockout models, we have provided a platform for understanding the nuanced interactions of these drugs with glioma cells, with potential implications for refining therapeutic strategies for glioma. Our study opens a window into the influence of EZH2 inhibitor and H3K27Me3 loss on glioma cells devoid of EZH2 expression. These findings offer vital insights for discerning which glioma subsets could be effectively treated with EZH2 inhibitors and the genes and pathways regulated by H3K27Me3 enriching our therapeutic strategies against these formidable cancers.

Society of Neuro-Oncology, Florida – November 2022

Defining the role of Histone H3 K27 methylation and H3.3 S31 phosphorylation in H3.3K27M mutant diffuse midline gliomas

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Diffuse midline gliomas (DMG) are aggressive high-grade gliomas that characteristically occurs in children. Heterozygous K27M mutation occurs in histone H3.3 as early events in DMG and are sufficient to drive a global reduction of H3K27 methylation (H3K27me) on all histone H3 variants. Histone H3K27me is responsible for epigenetic gene repression, and loss of K27me is associated with epigenetic dysregulation. However, histone H3.3 has a unique Chk1 phosphorylation site, Serine 31, critical for mitotic checkpoint regulation. Masking of S31 phosphorylation results in the abrogation of cell cycle checkpoints that monitor the alignment and segregation of chromosomes. DMG cells have decreased levels of mitotic S31P and are chromosomally unstable and aneuploid. Genomic-editing of H3.3 to revert the K27M mutation restores mitotic S31P and K27me and significantly reduces the rate of chromosome instability (CIN). Expression of H3.3 K27M or a non-phosphorylatable S31A mutant in normal, diploid cells results in chromosome missegregation and cell cycle checkpoint defects; and cells fail to undergo G1 cycle arrest leading to aneuploidy. H3.3 K27M and S31A inhibit p53 accumulation. In a mouse model of DMG, H3.3K27M and H3.3S31A promoted the development of high-grade gliomas, whereas H3.3WT controls did not. H3.3S31A is WT for K27me, demonstrating that loss of S31P is oncogenic. It remains unclear if it is the H3.3 K27M mutations or the associated loss of H3K27me3 that drives the reduction of S31P. The PRC2 complex is responsible for H3K27me, catalyzed by methyltransferases subunits EZH1 and EZH2. We used genome editing to knockout EZH1 and EZH2 in DMG cells and untransformed human diploid cell lines. Deletion of EZH2 causes a loss in K27 tri-methylation, and EZH1/2 knockouts causes a global loss of K27me. Ongoing studies examine how the loss of K27me affects gene expression, proliferation, H3.3S31P, chromosome missegregation, and aneuploid.

10th Annual Masonic Cancer Center symposium, Huntington Bank Stadium (formerly TCF Bank Stadium), University of Minnesota – May 2022

Coordinated disruption of H3K27 methylation and H3.3S31 phosphorylation orchestrates chromosome instability in DIPG

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Diffuse intrinsic pontine gliomas (DIPGs) are aggressive high-grade gliomas of the brainstem. With a median overall survival of only 9–15 months, they are among the most lethal of all human cancers. Heterozygous K27M mutations in histone variants H3.3 and H3.1 occur as early events in DIPG and are sufficient to drive a global reduction of H3K27 bi- and tri-methylation (me2, me3) on all histone H3 variants. Histone H3K27 methylation is responsible for gene repression and loss of methylation is associated with epigenetic dysregulation in H3.3K27M cells. H3.3 also has a unique Chk1 phosphorylation site, S31 that is critical for mitotic checkpoint regulation. Expression of H3.3K27M or a non-phosphorylatable H3.3S31A mutant in normal, diploid cells results in chromosome missegregation, cell cycle checkpoint defects, and failure to undergo G_1 arrest in response to missegregation, leading to aneuploidy. It is unclear whether gliomagenesis is induced by altered H3K27 methylation or loss of mitotic H3.3S31 phosphorylation, or both. Here using CRISPR Cas9 H3.3 mutant cell lines and knockouts of the PRC2 histone methyltransferase genes, EZH1 and EZH2, we test if either loss of H3K27 methylation or S31 phosphorylation contributes to mitotic defects. Quantitative immunofluorescence revealed that K27M cells have lower pS31 levels than WT H3.3 cells; consistent with our *in vitro* kinase data. The loss of pS31 was restored by knockout of the H3K27M allele. Interestingly, the total loss of K27me in EZH1/2 knockout cells caused an increase in pS31 levels. K27M, S31A, or loss of K27me correlates with mitotic defects. However, K27M and S31A display a different mitotic phenotype than EZH1/2 knockout cells. K27M and S31A cells had normal mitotic timing but often leave a chromosome in the cleavage furrow during anaphase. EZH1/2 double knockout cells have difficulty in aligning their chromosomes at the metaphase plate, resulting in extremely prolonged mitosis. The cohesin complex links sister chromatids from S-phase through anaphase onset. A cohesin fatigue assay revealed significantly reduced cohesin strength in these cells, resulting in weak sister chromosome pairing and difficulties in aligning chromosomes at the metaphase plate. This study shows that both loss of K27Me and pS31 generates mitotic defects through independent mechanisms. Further studies are needed to understand the molecular mechanism by which these changes influence mitosis in the context of cancer.

6th Neuro-Oncology Symposium on May 23-24th, 2022 at McNamara Alumni Center in Minneapolis, MN

Loss of Histone H3 lysine 27 methylation and H3.3 serine 31 phosphorylation causes chromosome instability and reduced cohesion strength in DIPG

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Diffuse intrinsic pontine gliomas (DIPGs) are aggressive pediatric high-grade gliomas with a median overall survival of only 9–15 months. Heterozygous K27M mutation occurs in histone H3.3 and causes a global reduction of H3K27 methylation and epigenetic dysregulation. Currently, no therapeutics promote survival for DIPG. Loss of H3.3 serine 31 (S31) phosphorylation in H3.3 K27M and non-phosphorylatable H3.3S31A mutant cells leads to chromosome instability (CIN), cell cycle checkpoint defects, and aneuploidy. CRISPR reversion of H3K27M allele to wildtype or expression of K27MS31E (phosphomimetic) restored these effects. However, it is unclear whether the K27M mutations or the global loss of H3.3 K27 methylation drives the loss of mitotic H3.3S31 phosphorylation and CIN. To address this, we use CRISPR Cas9 knockouts of the PRC2 histone methyltransferase genes, EZH1 and EZH2, to test how the loss of H3K27 methylation reflects on S31 phosphorylation and the mitotic phenotype. We found EZH1/2 knockout cells had misaligned chromosomes during metaphase with delayed mitosis and altered H3.3S31 phosphorylation. The cohesion complex coordinates sister chromatids pairings, homologous recombination**,** metaphase alignment, and smooth anaphase separation. The increase in mitotic timing led us to examine the strength of the cohesion complex on chromosomes. Quantitative immunofluorescence cohesion fatigue analysis showed reduced cohesion strength in EZH1/2 knockout cells, a hallmark of cohesion fatigue. It remains unclear how impaired H3K27methylation and a loss of H3.3S31 phosphorylation cause cohesion fatigue on chromatids and result in prolonged mitosis. Further studies are required to understand the molecular cues of this defective mitosis, which might lead to developing novel mitotic therapeutic targets for DIPG.