The role of H3.3K27M-induced gene repression in brainstem gliomagenesis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Diffuse Intrinsic Pontine Glioma (DIPG) is a highly aggressive pediatric brainstem tumor recently found to contain high incidence of H3.3K27M mutations. These mutations reprogram the H3K27me3 epigenetic landscape of DIPG by inhibiting the H3K27-specific histone methyltransferase EZH2. This leads to global reduction with focal gains of the repressive H3K27me3, a mark responsible for cell fate decisions. To date the tumor-driving effects of H3.3K27M remain largely unknown. We show H3.3K27M cannot form tumors alone, however it cooperates with PDGF signaling in vivo, enhancing gliomagenesis and reducing survival of p53 WT and knockout murine models of DIPG. We find H3.3K27M expression drives increased proliferation of tumor-derived murine neurospheres, suggesting cell cycle deregulation may contribute to increased malignancy in mutant tumors. RNA-Seq on tumor tissue from H3.3K27M expressing mice showed global upregulation of PRC2 target genes, and a subset of newly repressed genes enriched in regulators of development and cell proliferation. Strikingly, H3.3K27M induces targeted repression of the p16/ink4a locus, a critical regulator of the G0/G1 to S phase transition. We find increased levels of H3K27me3 at the p16 promoter, however pharmacological reduction of this promoter methylation does not rescue p16 expression. While DNA methylation is also present at this promoter, it is not K27M-dependent. Intriguingly, inhibition of DNA methylation restores p16
levels and is cytotoxic against murine tumor cells. Importantly, we show that H3.3K27M-mediated p16 repression is an important mechanism underlying the proliferation of H3.3K27M tumor cells as in vivo cdkn2a knockout eliminates the survival difference between H3.3K27M and H3.3WT tumor-bearing mice.
Dedication

I dedicate this body of work to the sacrifices of my mother and father, who left everything they knew to give my brothers and I infinite opportunity.
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1. Introduction

Diffuse Intrinsic Pontine Glioma (DIPG) is a rare yet extremely aggressive pediatric glioma localized to the pons region of the brainstem. Despite over 50 years of research dedicated to the development of an affective treatment for this deadly disease, still no therapy is able to improve survival past that of radiation alone. The median age upon diagnosis is between 6 to 7 years of age, and patients have a median survival of less than one year after being diagnosed, with less than 20% surviving past two years. The diffuse and infiltrating nature of this highly aggressive tumor, together with its sensitive location within the pons region of the brainstem, prevents any surgical resection. The biopsy of DIPG is an extremely sensitive procedure, which has only recently begun to be more commonly performed in the US. The only currently approved treatment is the standard regimen of radiation therapy, which only provides a temporary relief of symptoms and increases survival by just a few months.

Large scale sequencing efforts using patient tissue within the past decade have begun a new era of rapid discovery in DIPG biology as they elucidate potential driving mechanisms of this deadly disease. These large efforts have uncovered a set of the most commonly occurring genetic and epigenetic aberrations that are associated with DIPG. Earlier analyses discovered amplifications in Platelet Derived Growth Factor Receptor alpha (PDGFRA) and mutations or deletions in the TP53 are among the most frequent
genetic alterations in DIPG. More recent sequencing studies have discovered a novel and frequent mutation in H3 and H3.3 histone tails occurring at amino acid residue lysine 27 exchanging the residue for a methionine (K27M). This finding has become an intense area of focus due to the high frequency of DIPG patients positive for this mutation and its association with an even worse prognosis. The H3 and H3.3 histones are highly conserved, and the K27M mutation is the first mutation of a histone linked to cancer. Unraveling the role of this histone mutant and downstream pathways it affects may hold the key to the development of the first effective treatments for DIPG.

1.1 Brainstem glioma and DIPG

The human brainstem is comprised of 3 major regions, known as the midbrain, pons, and medulla, each with its own distinct and critical functionalities. The brainstem is especially delicate due to its role in serving as the bridge between the neocortex and spinal cord as well as maintaining basic vital functions such as heart rate and breathing. Gliomas arising in any compartment of the brainstem, also termed brainstem gliomas (BSGs) have been found in all midbrain, pons, and medulla compartments and are the leading cause of death in children with brain tumors. Earlier works hypothesized that gliomas of the brainstem were formed through similar mechanisms as cortical gliomas, however with the advent of high throughput sequencing, it is now evident that these tumors are distinct entities based on both location and age. BSGs seem to be linked to
early development as they occur primarily in children, were they account for 15-20% of all pediatric brain tumors\textsuperscript{4}. The median age of diagnosis for BSG is between 6 and 7 years old\textsuperscript{5}. Originally classified in 4 subgroups based on the dispersion of glioma cells combined with the region of occurrence within the brainstem, BSG is now considered to be a heterogenous group of tumors and categorized by treatment and prognosis. Group 1 includes the dorsally exophytic and cervicomedullary tumors which some evidence show significant benefit from surgical resection. Group 2 contains focal tectum glioma (solid or cystic) which shows some association with neurofibromatosis type I (NF1). Finally group 3, consisting of 80% of all BSGs, are the diffuse intrinsic pontine gliomas (DIPG), which have the worst prognosis, rapidly progressing symptoms, cranial nerve palsies, long track signs, and a median survival of less than one year upon diagnosis\textsuperscript{6}.

DIPG is considered a high grade BSG, with about 200-300 new cases diagnosed each year in the United States alone\textsuperscript{7}. Histology on DIPG tissue reveals these tumors to be GBM (WHO grade IV), anaplastic astrocytoma (WHO grade III), and less frequently well- differentiated diffuse astrocytoma (WHO grade II)\textsuperscript{8,9}. Currently the only approved therapy for DIPG is a standard regimen of radiation therapy, which provides only temporary relief of symptoms and has a median time to progression between 5-9 months\textsuperscript{10}. Until recently, the biopsy of DIPG was uncommon due to the presumed high
risk compared to the usefulness of the data that could be gleaned from a small sample of a heterogenous tumor\textsuperscript{11-13}.

Today, thanks to the work of several groups, led by a French research group, the safety, efficacy, and clinical utility of DIPG biopsy have improved and the practice is becoming more common\textsuperscript{14-17}. Consequently, thanks to increased high-quality sample accessibility within the past decade we have seen a wealth of knowledge emerge on the genetic and epigenetic alterations underlying DIPG biology. The early and subsequent studies discovered prevalent p53 loss or mutation in about 77\% of patients, as well as activation of the EGFR pathway through amplification or overexpression\textsuperscript{18-23}. Further studies identified amplification or activating mutations in the \textit{PDGFRA} locus in 36\% of DIPG\textsuperscript{3,24-28}. These genetic alterations were, until recently, thought to be the major contributors to DIPG gliomagenesis. Other alterations have been found, albeit much less frequently, in genes involved in the receptor tyrosine kinase- Ras- phosphoinositide 3-kinase (PI3K) signaling pathway\textsuperscript{29}. Additionally, alterations of genes regulating RB phosphorylation and cell cycle, amplifications of MYC/MYCN, and mutations in \textit{ATRX} and \textit{PPM1D} have also been found in DIPG samples\textsuperscript{30}. While p53 activity is repressed in the majority of DIPG patients, other tumor suppressors including \textit{PTEN} and rarely \textit{CDKN2A}, are also targets for genetic alteration\textsuperscript{23,31,32}. Data from our lab suggests there is
also epigenetic silencing of the CDKN2A transcripts, which we will discuss in further detail later in this work.

More recently, two new families of mutations have been discovered, one of which has gained the focus of not only the DIPG field but also epigenetics research. Firstly, mutations in the ACVR1 gene have been found in about 24% of DIPG patients\textsuperscript{39,33}. There are several variants of mutations of this gene found in DIPG, and interestingly some of which are the key driving mutation to the genetic disease fibrodysplasia ossificans progressiva (FOP), also known as stone man syndrome due to aberrant connective tissue ossification. The focus of this dissertation will be on the novel mutations discovered in the H3 histone protein tail residue lysine 27 (H3.3K27M), which have been found in up to 80% of patients and are thought to be a driving force in DIPG gliomagenesis\textsuperscript{34-36}.

\textbf{1.2 H3.3K27M inhibits PRC2 function}

The novel K27M histone missense mutations occur in a heterozygous fashion and are found in the canonical, replication-dependent H3.1, however they are more common in the non-canonical, replication-independent H3.3 histone proteins. Interestingly the K27M mutations are location dependent and primarily occur in midline tumors. This is important as a different mutation in the non-canonical H3.3 histone was concurrently discovered with a location favoring non-brainstem gliomas, suggesting a
cell-type specific effect. The histone mutation variants are all mutually exclusive with themselves as well as with IDH mutations. H3.3K27M has been shown to act as a potent inhibitor of the enhancer of zeste 2 (EZH2) histone methyltransferase component of the polycomb repressive complex 2 (PRC2) complex, however the exact mechanisms, which lead to enhanced gliomagenesis, are still unknown\(^{37}\). Interestingly, the H3.3G34R/V mutation has been shown contribute to gliomagenesis in part though the activation of \( MCYN^{38}\). The work presented in this dissertation begins to shed light on an important tumor-driving mechanism of the H3.3K27M mutations.

Some of the most commonly occurring somatic mutations occur in genes that encode for proteins functioning in chromatin dynamics\(^{39}\). PRC2 is a critical protein complex that regulates many cell fate gene regulators through the addition of repressive di- and tri- methylation of K27 on histone H3 and is also frequently associated with tumorigenesis\(^ {40,41}\). The repressive H3K27me3 mark is known to recruit PRC2 in a positive feedback loop to ensure silencing of the associated loci\(^ {42,43}\). PRC2-inducd silencing by H3K27me3 is caused by the recruitment of polycomb repressive complex 1 (PRC1) which contains H3K27me3 binding domains to identify silencing sites\(^ {44}\). Once bound to H3K27me3, PRC1 invokes chromatin compaction through its RING 1B and Bmi1 components\(^ {45}\). These catalyze H2A monoubiquitination, which enhances chromatin compaction as well as inhibits SWI/SNF complexes from opening chromatin
for gene expression. Therefore, PRC2 mediated H3K27me3 addition enables the silencing of chromatin through DNA compaction and is an important gene regulator. Lewis et al. first determined the effect of H3.3K27M on global H3K27me3 levels by using western blots for several methylated and acetylated histone marks. They found that the di- and tri- methylation of H3K27 was significantly reduced in human DIPG cells, transfected HEK 293 cells, as well as in our high-grade mouse model of DIPG. This reduction in H3K27me3 was more than expected from simply the loss of K27 on the K27M mutant H3.3 histones, which constitute only a small number of the more commonly expressed H3.1 histone.

Interestingly, Lewis et al performed substitution of K27 on histone H3 with all 20 amino acids and found that only isoleucine was able to mimic the dramatic loss of di- and tri-methylation of H3. Using intricate mononucleosome assays in which they measured EZH2 methyltransferase activity while titrating K27M –containing nucleosomes to WT H3, they found K27M acts as a potent inhibitor of PRC2 activity. This global reduction of H3K27me3 has become the recognized phenotype associated with H3.3K27M mutations. Further studies have suggested that K27M globally inhibits EZH2 by irreversibly binding the SET domain, sequestering it from methylating WT H3. Recent crystallography studies have shown strong evidence in favor of this interaction, as the PRC2 crystal structure was determined while bound to the mutated
H3.3K27M histone tail residue. The dramatic loss of H3K27me3 as a result of K27M inhibition of PRC2 activity has been shown to cause correspondingly large gene expression changes. This contributes to a unique gene expression profile distinguishing H3.3K27M expressing DIPG from other high-grade gliomas and may suggest differences in treatments. The H3.3K27M mutation also drives a DNA hypomethylation pattern and gene upregulation, however ChIP-Seq studies on DIPG patient samples and cell lines have discovered regions of focal gain of residual H3K27me3 and consequent gene repression. These focal increases in H3K27me3 have been shown to concentrate at the promoter regions and lead to local inhibition of genes critical to cancer pathways. Our mouse model of DIPG has shown a similar gene expression pattern and here we focus on further elucidating the importance of H3.3K27M-induced gene repression.

### 1.3 Roles of EZH2 in cancer

EZH2, the specific H3K27 histone methyltransferase component of PRC2 is intensely studied for its roles in both development and cancer. First identified in *drosophila* for its role, along with other polycomb proteins, in maintaining repression of the *HOX* genes to develop anterior-posterior body patterning. It is now known to be critical during development in regulating gene expression patterns to promote cell fate decisions. The role of EZH2 in cancer has been a complex problem as it has been shown to have both tumorigenic and tumor suppressor functions. Upregulation or
hyperactivation of EZH2 with subsequently increased H3K27me3 is known to occur in certain breast cancers, prostate cancers, and non-hodgkin lymphomas\textsuperscript{56-58}.

Studies have shown that upregulation of EZH2 contributes to increased proliferation, invasion, metastasis, and compromise DNA damage repair both \textit{in vivo} and \textit{in vitro}\textsuperscript{59-62}. It has also been shown to play a role in the inhibition of differentiation, maintaining cells in a primitive state and supporting cancer stem cells. In these roles EZH2 silences the expression of tumor suppressor genes. In addition to increased EZH2 activity contributing to tumor formation and maintenance, there are also several examples highlighting a potential tumor suppressive role.

Mutations or deletions directly or indirectly inhibiting either EZH2 or PRC2 function are also commonly found in several cancer types. \textit{JARID2}, \textit{EED}, and \textit{SUZ12}, are all critical components of PRC2 responsible for DNA binding and EZH2 stabilization, and are frequently mutated in many lymphomas and myeloid tumors suggesting an association with tumorigenesis\textsuperscript{63-66}. To add to this, depletion of EZH2 or other PRC2 components have been shown to cause enhanced lymphomagenesis in mice as well\textsuperscript{67}. Importantly, both EZH2 inactivation and over-activation have been shown to be sufficient for tumor formation\textsuperscript{68,69}. These opposing roles of EZH2 activity in different cancer types suggest a context-dependent role for PRC2. The context-dependent nature of EZH2 and its role in cancer is clearly highlighted by the lack of separation between
activating and deactivating EZH2 alterations in different tissues or tumor types\textsuperscript{70}. Additional data implies PRC2 mutations are contingent upon other requisite genetic alterations\textsuperscript{71-73}. Taken together, this suggests that the function of PRC2 as either a tumor enhancer or suppressor is reliant upon the genome of the cell of origin. Interestingly, Monje \textit{et al}, elegantly demonstrates a potential cell of origin for DIPG\textsuperscript{74}. This specific nestin-expressing cell population arises around the average time of peak DIPG diagnosis and has activated hedgehog signaling, a pathway known to be upregulated in patients. We utilize this knowledge in our mouse model of DIPG as we target the \textit{Nestin} expressing cells of the mouse brainstem to induce gliomagenesis.

\subsection*{1.4 DNA methylation in cancer}

DNA methylation is a covalent modification in which DNA methyltransferases (DNMTs) methylate the 5’ end of cytosines using S-adenosyl-methionine as a donor\textsuperscript{75}. This epigenetic mark is a long-term silencer of gene expression and occurs at CpG sites within the chromatin. There are 3 different DNMTs in mammals, DNMT1, DNMT3a, and DNMT3b, each with a slightly different methylating function. DNMT1 is the most highly expressed DNMT as it primarily functions in the critical maintenance of DNA methylation, however some studies suggest it can also catalyze \textit{de novo} methylation as well\textsuperscript{76}. The \textit{de novo} methylation of CpG islands refers to the addition of methyl groups to CpG sites devoid of methylation, usually CpG islands, which are large stretches of DNA
(-300bp) within the DNA containing about 50% CpG base pairs. DNA maintenance occurs in dividing cells, where DNMT1 binds to the daughter strand of DNA at a replication fork and methylates the newly synthesized DNA to match the parental strands methylation pattern. DNTM3a and DNMT3b are both de novo DNA methylators that serve non-overlapping functions and are essential for the establishment of DNA methylation patterns after early implantation\textsuperscript{77}. All three DNMTs are critical in establishing gene expression patterns to support normal development, and can contribute to tumorigenesis when their DNA methylation patterns are altered.

Interestingly, DNA methylation is completely removed and reestablished after the zygote has implanted into the uterine wall of the mother. Once reestablished, the resulting global DNA methylation patterns are stable regulators of gene expression and are maintained throughout all subsequent DNA replications and cell divisions. Importantly in normal development most CpG islands are protected from methylation as they are commonly localized near transcription start sites and transcription factor binding sites, thus allowing for activation of the downstream gene upon activation\textsuperscript{78}. Studies comparing the methylation of CpG islands have exposed intricate links between the early establishment of DNA methylation and PRC2 regulated H3K27me3 deposition.

Evidence shows a strong negative correlation between areas enriched with DNA methylation and those with PRC2 mediated H3K27me3. Studies have provided evidence
showing that demethylated CpG sites can recruit PRC2 silencing\textsuperscript{79,80}. Furthermore, bisulfite high-throughput sequencing and H3K27me3 ChIP-Seq analyses support that the DNA methylome negatively correlates with H3K27me3 patterns both in normal and cancer tissues\textsuperscript{81-86}. More compelling data supporting crosstalk between DNA methylation and PRC2 silencing has come from DNMT knockout (KO) studies. Work by Reddington et al using DNMT1 KO mice showed that the dramatic hypomethylation caused by loss of DNMT1 led to a redistribution of the H3K27me3 pattern\textsuperscript{87}. Interestingly, in the DNMT1 KO mice PRC2 and H3K27me3 deposition was shifted to reflect the normal, DNMT1 WT DNA methylation pattern. This led to loss of both PRC2 and H3K27e3 at PRC2 target genes, leading to the aberrant activation, mimicking loss of PRC2 repression. A similar crosstalk may be occurring in H3.3K27M mutant cells as the global loss of H3K27me3 may subsequently lead to changes in the DNA methylome.

DNA methylation analysis of tumor cells in many different cancer types have revealed that there are irregular DNA methylation patterns including the \textit{de novo} methylation of some CpG islands\textsuperscript{88,89}. However, many of these newly methylated CpG sites have been shown to be from a large variety of gene types and not singularly associated with cell growth and cancer\textsuperscript{90}. Interestingly, these sites of \textit{de novo} methylation are associated with CpG islands that are marked with PRC2, suggesting these systems have crosstalk mechanisms in place\textsuperscript{91-93}. Indeed there is strong evidence that the DNA
methylation and PRC2 silencing pathways are mechanistically linked. Studies using immunoprecipitation and ChIP-qPCR have demonstrated binding between the PRC2 components EZH2 and EED, with all 3 DNMTs. Therefore it is possible that sites with H3k27me3 could recruit DNMTs and subsequent DNA methylation for additional silencing in malignant gliomas. The interplay between PRC2 and DNA methylation in the context of H3.3K27M mutation remains understudied, however these interactions could be important in the observed H3.3K27M-induced silencing.

1.5 Modeling H3.3K27M brainstem glioma

The rarity of DIPG and subsequent scarcity of patient samples enhances the importance of the development of accurate models in order for in depth study of this disease. Currently there are 3 main categories of animal models, each with advantages and disadvantages.

Stereotactic implantation of glioma cells into the rodent brain has been a widely used tool for glioma research, although these systems have been better established for cerebral cortex glioma models. The first demonstration that heterotopic cells could grow in the rodent brainstem came from the injection of human medulloblastoma cells into the cisterna magna of nude rats leading to tumor cell colonization in the medulla and pons. This suggested that modeling glioma in the brainstem of rodents could be a possible experimental approach for studying the biology and treatment of DIPG. The
first animal models developed for DIPG specifically involved the intracranial injection of rat glioma cell lines, F98, 9L, or C6 into the brainstem of neonatal or adult rats leading to the generation of brainstem glioma\textsuperscript{97,98}. However, the tumor cells had been derived from adult gliomas that arose in the cerebral cortex of rats and had been heavily passaged in culture. Therefore, although these models did take into account the specific microenvironment of the brainstem, any innate differences between cerebral cortex and brainstem glioma cells were ignored.

Next, several groups generated human xenograft models in which human adult cerebral cortex glioblastoma cells, either from cell lines or serially transplanted xenografts, were transplanted into the brainstem of rats or mice, leading to tumors histologically and anatomically resembling human DIPG\textsuperscript{99-101}. As these tumors were composed of human glioma cells growing within the brainstem, these models were designed for the purpose of investigating therapeutic response rates, taking into account the unique microenvironment and blood-brain-barrier of the brainstem.

Given the limitations of allogenic orthotopic models discussed above an important step in the field came when the first group developed human DIPG-specific cell and xenograft lines derived from autopsy material of a pediatric brainstem glioma\textsuperscript{74}. DIPG autopsy tissue was cultured \textit{in vitro} in neural stem cell conditions that supports the growth of tumor neurospheres expressing (to varying degrees), Nestin, GFAP,
Vimentin, Sox2, Olig2, and CD133, suggesting a primitive neural precursor cell type. Stereotactic transplantation of dissociated neurospheres into the fourth ventricle of immunodeficient neonatal mice led to the development of tumors in the hindbrain, diffusely infiltrating the brainstem, cerebellum, and cerebrum, with histopathology reminiscent of DIPG. In contrast to working with autopsy material, models of DIPG using tissue harvested from a living biopsy is beginning to emerge, as the practice of biopsy is becoming more common. Several groups have developed human DIPG cell lines from tumor samples harvested at diagnosis during biopsy procedures. These lines have been used to study DIPG biology as well as therapeutic testing.46,102-104

Others have implanted DIPG cells collected from biopsy into mice after in vitro neurosphere culture. These studies provide important precedent that naïve DIPG cells from biopsy specimens are able to grow in vitro and be propagated in immunocompromised mice in vivo. Future work in this system should be expanded upon in the future to test potential therapeutic agents or delivery mechanisms. Although biopsy tissue represents a small sample of the tumor and may not be representative of the entire tumor, these types of models will perhaps be more predictive of therapeutic response rates in patients than models based on autopsy material, as the cells have not been previously subjected to treatment. Regardless, these human DIPG-derived cell and xenograft models are much improved over the initial
allograft rodent models, as they are composed of human DIPG cells and are growing within the unique microenvironment of the brainstem.

An important complement to human xenografts in mice is the use of genetically engineered models, as these systems are driven by a specific set of genetic alterations introduced in a particular cell-of-origin. The establishment of such models was delayed, however, by a lack of knowledge regarding the genetic drivers of DIPG. The increased understanding in recent years of the underlying genetics of DIPG provides a starting point for genetically modeling these tumors in the lab.
Genetically engineered mouse models (GEMMs) provide the advantage of studying tumors that arise in their natural microenvironment in immune-proficient animals. Our lab has generated the first GEMMs of DIPG utilizing the RCAS (replication-competent avian sarcoma-leucosis virus long terminal repeat with splice acceptor)/TVA (Tumor virus A) modeling system and genetic alterations commonly found in the human disease (Figure 1)\textsuperscript{37,106,107}. The RCAS-Tva system uses the retroviral avian leucosis and sarcoma virus family as a gene delivery vector. This virus exclusively infects cells expressing the corresponding surface receptor TVA, normally expressed in avian cells. Genetically engineered mice have been generated to express the TVA receptor under the control of several cell-type specific promoters\textsuperscript{108}.
Figure 1: RCAS GEMM of DIPG
Using these mice, the RCAS system revealed gliomas could be generated targeting cells outside of the subventricular zone\textsuperscript{109}. This finding showed the potential of the system to be used for modeling DIPG.

Early work with the RCAS system used germline Ink4a-ARF loss and PDGFB overexpression targeted to Nestin-expressing cells of the neonatal mouse pons, the likely cells-of origin for DIPG\textsuperscript{74,107}. Tumors arising in this model were located within the murine brainstem and were histologically similar to human DIPG, however they were not exclusive to the pons region of the brainstem. Therefore this system is described as a brainstem glioma (BSG) GEMM and additional, more spatiotemporally accurate, models are needed for DIPG. Identification of pons-specific promoters or enhancers would allow for the generation of mouse strains that drive expression of Tv-a (for use with the RCA/Tv-a system), Cre, or DIPG-specific oncogenes. Alternatively, pons-derived stem cells can be isolated, cultured and infected by RCAS viruses \textit{in vitro}, and re-introduced into the murine pons as an \textit{ex-vivo} approach although the \textit{in vitro} culture conditions will alter the cells.

Additionally, our lab was the first to use GEMMs to generate high-grade BSGs and test for therapeutic response using radiation and perifosine, an inhibitor of AKT signaling\textsuperscript{107}. They found a survival benefit using a 10Gy radiation dose, however no additional benefit was seen using the combined radiation with perifosine treatment.
However, in another study using this early GEMM, we demonstrate a survival benefit with the CDK4/6 inhibitor PD-0332991 alone and in combination with radiation\textsuperscript{106}. Importantly, this was the first demonstration of any targeted agent prolonging survival over radiation therapy alone in a pre-clinical setting. This early BSG GEMM showed that modeling glioma in the mouse brainstem was possible and was pivotal in the identification of a promising new therapy for DIPG.

RCAS-based GEMMs have evolved by utilizing the increasing understanding of DIPG genetic alterations, incorporating the three most highly recurring genetic alterations: PDGF signaling overexpression, p53 loss, and the H3.3K27M mutation\textsuperscript{37}. Cells producing the RCAS-PDGFB, RCAS-Cre, and RCAS-H3.3K27M viruses were delivered into the brainstem of Nestin-Tva; p53-floxed neonatal mice and resulted in high-grade BSG with gene expression similar to human data\textsuperscript{37}. This model was the first to show global loss of H3K27me3 levels with expression of the K27M mutation, similar to what is seen in patient samples. The DIPG pre-clinical consortium recently used this GEMM to test the promising new drug BMS-754807, a potent multi-kinase inhibitor\textsuperscript{110}. So far, no survival benefit has been found using the novel BSG GEMM, but Halvorson et al. showed promising \textit{in vitro} data and found the drug concentration levels in treated mice were well below the know IC-50 for BMS-754807. The aggressiveness of this most
recent BSG GEMM parallels the clinical disease and represents a good tool for pre-clinical therapeutic trials.

In addition to being used as a pre-clinical tool to test potential therapeutics for DIPG, GEMMs can be used to study the biology of the disease in an *in vivo* setting, which may help to uncover novel characteristics of the human disease. In this regard, using the RCAS model described above, comparison of gene expression profiles of BSGs versus those initiated in the cerebral cortex revealed that those in the brainstem harbored high levels of the transcription factor Pax3\textsuperscript{111}. *In vitro* and *in vivo* studies suggest that overexpression of this gene contributes to the gliomagenesis process by inhibiting apoptosis and promoting proliferation. Analysis of human tumor cohorts showed that 40% of DIPG patients’ tumors are characterized by particularly high levels of Pax3, which associates with activation of PDGF signaling, amplification of cell cycle regulatory genes, and is exclusive of ACVR1 mutations. This work further defines a subset of human DIPG and lends insight into novel mechanisms driving tumorigenesis.
2. H3.3K27M in normal neuronal progenitors

We begin our functional study of H3.3K27M by first asking whether it is sufficient to induce brainstem glioma in our model without the co-expression of any other genetic alterations. This represents the first study examining the tumor forming capabilities of K27M alone and is an important step in our understanding of the pro-tumor mechanisms responsible for its impact on DIPG aggressiveness. Here we use both in vivo and in vitro approaches to analyze the impact of H3.3K27M on normal murine brainstem progenitors. Additionally, we employ ChIP-Seq and proteomics approaches in order to analyze the global changes imparted by H3.3K27M expression and determine whether there are malignant or pre-malignant signatures.

2.1 Reduced neuronal differentiation in H3.3K27M progenitors

RNA-seq studies have shown that many pathways involving differentiation are altered in H3.3K27M DIPG samples. Additional work has shown that H3.3K27M increases bivalent gene loci as well as immature stem cell characteristics. These results suggest K27M may inhibit neuronal maturation, contributing to the observed increased tumor aggressiveness in the human disease. To begin examining the impact of H3.3K27M in neuronal differentiation, we utilized our in vitro isolated neuronal progenitors infected with 1.) H3.3K27M, 2.) H3.3WT, or 3.) Empty vector cultured as neurospheres to perform differentiation assays. Normal and malignant progenitor cells cultured in serum-free conditions grow as non-adherent neurospheres that retain tumor
or neural progenitor characteristics and are commonly used to unravel mechanisms in stem cell biology\textsuperscript{112}. We used the addition of 10% FBS containing media to induce differentiation in order to examine the differentiation potential of cultured progenitors groups\textsuperscript{113}. We found that the expression of H3.3K27M in normal neonatal brainstem progenitors significantly reduces mature, TUJ1-expressing neurons, compared to empty vector, and H3.3WT controls (p-value= 0.001, and 0.026, respectively) suggesting there is a neuronal lineage maturation defect (Figure 2). This was an interesting finding as K27M mutant gliomas are subcategorized in the proneural glioma group, indicating a defect in maturation of a neuronal progenitor cell of origin\textsuperscript{48}. Proneural subtype gliomas are defined as having amplifications/mutations in \textit{PDGFRA} and display a gene signature containing several genes expressed during neuronal development such as Olig2\textsuperscript{114}. This leads to the exciting possibility that the K27M mutation may be directly inhibiting neuronal maturation in favor of a more neuronal progenitor-like state.
Figure 2: Decreased neuronal maturation

Isolated neuronal progenitors were infected with either empty vector (RCASY), H3.3WT, or H3.3K27M viruses, and incubated for 7 days with differentiation media containing 10% FBS. After incubation we performed double IF for TUJ1 (red), and (DAPI), and quantified the 40x images using ImageJ software.

2.2 H3.3K27M cannot induce glioma as a single factor

Using our RCAS-Tva murine model we next asked whether expression of H3.3K27M as a single factor is able to promote gliomagenesis in nestin-expressing progenitor cells of the murine brainstem. To begin our in vivo investigation of this, we injected either RCAS-H3.3K27M or RCAS-H3.3WT viruses into the brainstem of neonatal mice (postnatal day 3-4) and monitored for tumor symptoms. We allowed 12
weeks of incubation before sacrificing the mice to analyze for tumor development. All mice in both the H3.3K27M, and H3.3WT only groups survived up to the arbitrary 12-week end-point with no tumor symptoms (weight loss, increased head size, neurological symptoms). For our murine tumor studies we choose 12 weeks as a cutoff point as by this time tumors in our high-grade model have either caused sacrificing criteria in the mice or the injection was unsuccessful leading to no tumor initiation. H&E staining of formalin fixed, paraffin embedded (FFPE) tumor sections revealed no glioma or pre-malignant abnormalities at the brainstem injection site. Immunohistochemical analysis for the HA-tagged H3.3K27M, and H3.3WT histones revealed a small population of HA-positive cells remained near the brainstem injection area after 12 weeks, however double immunofluorescence (IF) for proliferating cell nuclear antigen (PCNA) showed these cells were not proliferating. We therefore conclude that expression of H3.3K27M as a single factor is not sufficient to induce gliomagenesis alone.

We next extracted normal nestin-expressing brainstem progenitor cells from the neonatal mice to grow in culture as neurospheres for further in vitro analysis of H3.3K27M expression alone. We collected and concentrated the virus-containing supernatant of our RCAS vector-infected chicken fibroblast viral producing cells and infected the in vitro brainstem progenitors with either RCAS H3.3K27M, H3.3WT, or empty vector viruses tagged with HA. We first examined whether the expression of the H3.3K27M mutation alone in our in vitro cultured nestin-expressing neural progenitor
cells is able to induce global loss of H3K27me3 after 2 passages (21 days) post-infection. To this end we performed histone extraction followed by western analysis to blot for 1.) H3K27me3, 2.) HA tag to control for RCAS construct expression, and 3.) Total H3 as a loading control. Interestingly, although we found similar HA signal indicating little variation between the mutant and WT H3.3 HA tagged exogenously expressed histones, the levels of H3K27me3 were equal among H3.3K27M, H3.3WT, and empty vector expressing neurospheres (Figure 3A,B). This is in contrast with data that others and we have published showing significant global loss of H3K27me3 and may be a consequence of cell type\textsuperscript{46,115}. Additionally, we examined proliferation differences between the groups using BrdU incorporation. In agreement with our \textit{in vivo} studies, our results showed no proliferation differences between the 3 groups by BrdU incorporation assays (Figure 3C).

Finally, we employed a ChIP- Seq strategy on the single virus infected neuronal progenitor cells to examine whether H3.3K27M changed the H3K27me3 and H3K4me3 landscapes throughout the epigenome compared to H3.3WT, and empty vector controls. For this we isolated fresh neuronal progenitor cells from neonatal nestin-tva mice, infected with concentrated H3.3K27M-HA, H3.3WT-HA, or empty vector RCAS viruses, and incubated for 2 passages. We then fixed the neurospheres in 1% PFA for 7mins, quenched the fixation with 125mM glycine, before flash freezing the cell pellet and submitting the cells to Zymo research for ChIP-Seq analysis.
Figure 3: No global loss of H3K27me3 or change in proliferation in progenitors with K27M mutation alone

A.) Histone extraction western blot of neuronal progenitor cells. B.) Quantification of western blot using ImageJ software. C.) Quantification of BrdU proliferation assay

We found good H3K4me3 and H3K27me3 ChIP signals from positive controls GAPDH and Hoxa11 primer sets, respectively, indicating the pull downs were efficient. We then analyzed the levels of the activating H3K4me3 and repressive H3K27me3 marks at specific gene structural elements important for regulating gene expression including: 1.) Promoter, 2.) 5’- UTR, 3.) Coding exon, 4.) Intron, 5.) 3’- UTR, and 6.) Intergenic regions. We found no change in H3K4me3 at all sites analyzed, which is in support of what studies using DIPG samples have observed\textsuperscript{46}. Surprisingly, the
H3.3K27M group showed a slight decrease in H3K27me3 compared to the empty vector control group, however the H3.3WT group exhibited the greatest loss at all sites measured (Table 1). Nevertheless, our data shows that expression of H3.3K27M in the neonatal brainstem progenitors is insufficient to drive gliomagenesis in our mouse model. Further analysis is needed to determine whether H3.3K27M alone can significantly alter the H3K27me3 landscape as a single factor.

Table 1: H3K4me3 and H3K27me3 gene structural element

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2.3 Proteomic analysis of K27M

As K27M mutation showed no impact on proliferation or H3K27me3 levels as a single factor in the nestin expressing neuronal progenitors, we used unbiased proteomics analysis to determine whether there were any protein level changes that could indicate a pre-malignant state. Using freshly extracted neuronal progenitor cells, we again infected with 1.) H3.3K27M, 2) H3.3WT, or 3.) empty vector RCAS viruses and incubated for 2 passages before collecting the cells and submitting them for unbiased
mass spectrometry and phosphor protein analyses. Results from the unbiased and phospho proteome mass spectrometry analysis showed that expression of H3.3K27M led to a unique signature as compared to control (Figure 4).

Figure 4: PCA of z-score corrected protein intensities or phosphoprotein intensities

Comparison analysis of the phosphorylated proteins revealed 40 phospho proteins were increased and 39 were decreased in the H3.3K27M group compared to the H3.3WT controls. Comparison of total proteins showed that 32 genes were increased and 55 were decreased between H3.3K27M and H3.3WT (table 2).

Table 2. Total significantly changed phospho- or unbiased proteins

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<td>Decreasing</td>
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We next performed gene ontology to better understand the pathways affected by expression of H3.3K27M using Panther software. In the phosphoprotein analysis, we found the top 3 pathways gaining phosphorylation in H3.3K27M cells vs WT were 1.) Adrenaline and nonadrenaline biosynthesis, 2.) corticotropin releasing factor receptor signaling pathway, and 3.) Dopamine receptor mediated signaling pathways. Interestingly the top pathway associated with the proteins showing decreased phosphorylation was in the apoptosis signaling pathway. Even more interestingly, gene ontology of the total proteome hits showed that only 2 pathways were increased in the H3.3K27M mutants compare the H3.3WT controls, the endothelin and vasopressin signaling pathways. Several pathways were associated in the proteins decreased in H3.3K27M vs WT, however there were no pathways with clear relevance to tumorigenesis. Taken together, the expression of H3.3K27M alone does change the proteomic signature however how these changes contribute to glioma progression remains to be studied.

2.4 H3.3K27M induces proliferating cells in vivo with p53 loss

Since expression of H3.3K27M alone was not able to induce gliomagenesis, we next paired the mutation along with a p53 floxed allele to induce knockout, as this
combination of genetic alterations commonly co-occurs in DIPG patients. We co-injected either 1.) H3.3K27M + Cre or 2.) H3.3WT + Cre virus producing cells in equal amounts into the brainstem of neonatal mice. All mice survived up to the 12-week endpoint without tumor symptoms. However, H&E sections of the H3.3K27M + p53KO mouse brains revealed the presence of small ectopic clusters of cells within the brainstem injection region in 72% (21 out of the 29). In contrast this was not observed in any of the H3.3WT + p53 KO control mouse brains (n=8).

Figure 5: Ectopic proliferating clusters in H3.3K27M brainstems with p53 KO
5 micron brainstem sections of mice, 12 weeks post-infection with H3.3K27M and Cre viruses for P53 knockout in nestin-expressing brainstem progenitors. IF images of A.) DAPI, B.) HA-tag, and C.) PCNA were taken at 40x and D.) Merged to analyze overlap.

We next use double immunofluorescence imaging (IF) using antibodies against proliferating cell nuclear antigen (PCNA), a marker of proliferating cells, and hemagglutinin (HA), which is fused to our H3.3K27M and WT histone constructs. This enabled us to examine whether the observed clusters were expressing the histone constructs and whether these cells were aberrantly proliferating. Here we found both H3.3K27M + p53 KO and H3.3WT + p53 KO groups contained positive HA staining indicating the presence of the exogenous histones within the ectopic cell clusters (Figure 5A-D). Notably, only the H3.3K27M mutant groups showed expression of PCNA, suggesting the mutant is needed to promote proliferation in vivo. However, this ectopic proliferation is not enough to produce gliomas.

Interestingly, we performed the in vitro equivalent of this experiment using extracted murine neuronal progenitor cells infected with either 1.) H3.3K27M + Cre, 2.) H3.3WT + Cre, and 3.) empty vector + Cre, and found that there was no difference in proliferation by BrdU analysis (Figure 6). This suggests that the proliferative effect of H3.3K27M expression requires the brainstem microenvironment, which may exhibit growth factors needed to stimulate H3.3K27M-induced increased proliferation. Additionally the majority of the proliferating cells within the clusters seem to devoid of
mutant expression, which may indicate a non-cell autonomous mechanism which should be further explored.

Figure 6: in vitro p53 KO cell proliferation
3. H3.3K27M accelerates gliomagenesis

Identifying the role of H3.3K27M-induced PRC2 inhibition on gliomagenesis has become an intense area of research as the prevalence and association with decreased survival of these mutations suggest they may be key drivers of DIPG\textsuperscript{10}. Several studies performing high-throughput analyses have produced a wealth of data showing the far-reaching influence of H3.3K27M on gene expression and the epigenetic landscape\textsuperscript{35,46,48}. This has set the stage for in-depth investigations into the mechanisms responsible for H3.3K27M-enhanced gliomagenesis. We utilized our mouse model of DIPG driven by PDGF signaling alone or with p53 KO to delve deeper into the phenotypes underlying H3.3K27M gliomagenesis using both \textit{in vivo} and \textit{in vitro} assays.

3.1 PDGF signaling supports H3.3K27M accelerated gliomagenesis

We turned to early clinical analyses using DIPG patient data revealed that H3.3K27M expression correlates with \textit{PDGFRA} amplifications and decreased overall survival when compared to patients lacking the histone mutation\textsuperscript{34}. As our early studies exploring H3.3K27M expression alone in neuronal progenitor cells revealed it is not sufficient to initiate glioma, Recent works have provided evidence suggesting expression of the H3.3K27M mutant histone in the presence of constitutively active PDGFRA leads to increased proliferation and stem cell characteristics \textit{in vitro}\textsuperscript{116}. Taken together this suggests H3.3K27M mutations may need other tumor driving factors such
as increased PDGF signaling in order to exert its tumor enhancing effects. Therefore we asked whether PDGF signaling and the H3.3K27M mutation act in concert to enhance gliomagenesis utilizing the RCAS/tv-a system.

PDGFB overexpression alone in our nestin tv-a murine model normally induces low-grade brainstem gliomas only detectable after histological analysis\textsuperscript{107}. This system enables for analysis of enhanced tumorigenesis with expression of H3.3K27M. We began our \textit{in vivo} experiment by examining the combined expression of PDGFB with H3.3K27M, H3.3WT, or empty vector (RCASY) to determine the impact of H3.3K27M on survival and tumor grade.

![Figure 7: Survival of PDGFB-driven mouse tumor groups](image-url)
The mice were injected with 1µL containing 100,000 cells of a 1:1 mix of either 1.) RCAS-PDGFβ + RCAS-H3.3K27M-HA 2.) RCAS-PDGFβ + RCAS-H3.3WT-HA or 3.) RCAS-PDGFβ + RCASY (empty vector) virus producing cells. All PDGFβ; empty vector control mice survived to the 12-week endpoint with no tumor symptoms. Similarly, the majority of the PDGFβ; H3.3WT control mice survived to the 12-week endpoint. Interestingly, 22% of the PDGFβ; H3.3K27M expressing mice developed tumor symptoms before the 12-week endpoint and met our criteria for sacrifice, showing a significant difference in survival as compared to empty vector controls (Figure 7, p=0.048). Histological analysis of the PDGFβ; empty vector, and PDGFβ; H3.3WT brains showed only grade II tumors. However, the PDGFβ; H3.3K27M cohort demonstrated 47% grade II, 47% grade III and 6% grade IV tumor incidences. The grade III-IV gliomas within the PDGFβ; H3.3K27M group displayed increased microvascular proliferation, mitotic figures, and overall increased cellular density as detected by H&E staining (Figure 8A, B). We injected EdU 4 hours prior to sacrifice to a cohort of PDGFβ + H3.3K27M and PDGFβ + H3.3WT mice to analyze proliferating tumor cells. Using double IF we found that the PDGFβ; H3.3K27M tumors contained a significantly higher percentage of EdU-positive cycling cells as compared to the PDGFβ + H3.3WT and PDGFβ + empty vector controls (Figure 8C). We additionally performed MRI scans on a subset of all three groups (PDGFβ; H3.3K27M n=6, PDGFβ; H3.3WT n=6, PDGFβ; RCASY n=7), just before the time of sacrifice to analyze tumor size. Tumor volume
analysis revealed a trend for larger tumors with the combined PDGFB + H3.3K27M expression and although noisy, complemented histological results (figure 8D). Thus, H3.3K27M increases proliferation, raises tumor grade, and reduces survival of PDGFB driven, p53 WT brainstem gliomas in mice.

Figure 8: Cooperation between PDGF signaling and H3.3K27M mutation in vivo

A.) H&E and IF images of PDGFB driven mouse brainstem glioma groups. B.) Quantification of tumor grades. C.) Quantification of percent EdU positive cells in IF images of PDGF-driven mouse tumor groups. D.) Quantified MRI tumor volumes
3.2 H3.3K27M accelerates gliomagenesis in a high-grade model

We next asked whether H3.3K27M increases the aggressiveness of our PDGF-driven, p53 KO, high-grade tumor model. To test this, we generated a high-grade murine model of DIPG by utilizing a nestin-tva mouse with a p53-floxed allele. We first examined survival between H3.3K27M and H3.3WT expressing high-grade tumors after injection of either PDGFB; H3.3K27M; Cre, or PDGFB; H3.3WT; Cre virus-producing DF1 cells into the brainstem of neonatal mice. We used a power analysis formula in order to determine the number of mice required to detect significant differences in survival between H3.3K27M and H3.3WT control mice\(^\text{117}\). Using this formula we determined that a study of 40 mice per group would be sufficient to detect a 5-day survival difference based on a standard deviation of 5 days in our HA-tagged model. This combination produced high-grade tumors, in which the mice presented with tumor symptoms in 4-6 weeks post-injection.

We found that H3.3K27M-HA mice exhibited significantly shorter tumor latency with a median survival of 36 days compared to 41 days in the H3.3WT-HA group (p=0.0065). We compared this to the survival in GFP-tagged H3.3 constructs that showed similar results, with H3.3K27M mutant mice succumbing to tumor an average of 36 days post-injection as compared to the H3.3WT-GFP mice, which had an average survival of 44 days (Figure 9, p=0.03). Interestingly, analysis of H3.3WT-HA and H3.3K27M-HA
tumors showed no difference in tumor cell proliferation by phosphorylated H3 (pH3) IHC or EdU immunofluorescence (Figure 10).

![Figure 9: Survival comparison of HA- and GFP- tagged H3.3 constructs in High-grade glioma model](image)

This may be a consequence of our experimental design, in which we collected the brains after the mice had reached our sacrificing criteria, indicating the tumor had reached a critical mass. At this point it is possible that both H3.3K27M and H3.3WT tumors have reached a maximum proliferation percentage. Therefore, further in vitro
proliferation analysis of these cells is required to determine whether there is increased proliferation with H3.3K27M expression in the high-grade model.

For this we utilized the GFP tagged model to purify primary tumor cells using fluorescence-activated cell sorting (FACS) to isolate the GFP-tagged H3.3WT or H3.3K27M populations from the tumor bulk. We cultured the purified cells in serum-free, neurosphere conditions as this method has been shown to better maintain the gene expression profile of the original brain tumor tissue\textsuperscript{112}. We first tested whether H3.3K27M induced a global reduction of H3K27me3 levels by histone extraction followed by western blot. Indeed, we found that cells expressing H3.3K27M showed a significant reduction in global H3K27me3 compared to the H3.3WT control tumor neurospheres (Figure 11A). Additionally, BrdU incorporation assays show that \textit{in vitro}
cultured H3.3K27M tumor neurospheres did exhibit significantly increased proliferation as compared to the H3.3WT-GFP control neurospheres (Figure 11B).

Figure 11: in vitro high-grade tumor cells exhibit decreased H3K27me3 and increased proliferation

A.) Histone extraction western blot of GFP-sorted in vitro high-grade tumor cells. B.) BrdU incorporation assay quantification. C.) Cell cycle analysis of propidium iodide stained cultured tumor cells (** < 0.001, * < 0.05)

Furthermore, we stained GFP-sorted H3.3WT and H3.3K27M lines with propidium iodide for cell cycle analysis and found a significant shift in the H3.3K27M lines toward the S and G2/M phases with a corresponding reduction in the G0/G1 phase cells (Figure 11C). This further indicates a higher percentage of proliferating cells in
H3.3K27M mutant lines. Together these results complement our data from the p53 WT experiments and support that expression of H3.3K27M leads to increased tumor cell proliferation by cooperating with PDGFB driven brainstem gliomas independently of p53 activity.
4. H3.3K27M alters PRC2 target gene expression patterns

Studies using DIPG patient samples have used RNA-Seq, ChIP-Seq, and bisulfite sequencing to analyze gene expression and epigenetic changes resulting from H3.3K27M mutations\(^4\). These studies have begun to shed light on the pathways that may be contributing to the increased aggressiveness imparted by H3.3K27M expression. However, patient data can be highly variable due to several factors including post-radiation treatment, tumor heterogeneity, and pre- or postmortem tissue collection. Additionally, age and potentially different cell of origins may also be contributing to masking differences in important pathways. To control for these factors, we utilize our DIPG mouse model to analyze the gene expression effects of the H3.3K27M mutation and determine potential key tumor-driving mechanisms contributing to the accelerated gliomagenesis.

4.1 H3.3K27M induces large-scale upregulation with focal repression of PRC2 targets

In order to begin to identify potential mechanisms underlying H3.3K27M-induced increased malignancy and cell cycle progression we began by analyzing gene expression changes using RNA-Seq. We generated tumors in our high-grade glioma mouse model and extracted tumor tissue from PDGFB; p53 KO; empty vector control and PDGFB; p53 KO, H3.3K27M expressing mice and subsequently performed RNA-Seq analysis. We found a total of 224 significantly differentially expressed genes when we
compared the H3.3K27M results to the empty vector tumors. As expected due to the loss of the repressive H3K27me3 mark, the majority of the significantly altered genes showed increased expression. We found 182 genes were upregulated and 42 were downregulated in the H3.3K27M group compared to the empty vector controls (Figure 12).
Unsupervised hierarchical clustering and principal component analysis revealed that the H3.3K27M group tumors exhibit a distinct gene expression profile compared to the PDGFB; p53⁻/⁻; empty vector controls (Figure 13A). Gene set enrichment analysis (GSEA) demonstrated that our RNA-seq results have a significant correlation with PRC2
regulated genes from embryonic and neural progenitor cell data, suggesting that the changes in gene expression are due to altered PRC2 silencing (Figure 13 B, C).

**Figure 13: H3.3K27M induces unique gene expression patterns regulated by H3K27me3 target genes**

A.) PCA plot comparing high-grade H3.3K27M tumors to empty vector controls. B.) GSEA enrichment plots comparing H3.3K27M mouse tumor RNA-Seq data to mouse embryonic fibroblast, and C.) Neural progenitor cell PRC2 gene silencing patterns

Gene ontology (GO) analysis using DAVID software indicated that the upregulated genes were enriched in neuronal function and morphogenesis pathways (Figure 14A). This is consistent with previous studies, which have shown H3.3K27M- induced upregulated genes are enriched in neuronal function and maturation48.
Intriguingly, known PRC2 target genes were also among the downregulated genes in H3.3K27M tumors, further indicating the presence of residual PRC2-induced repression despite H3.3K27M-induced inhibition of EZH2 methyltransferase activity. GO analysis of the downregulated genes revealed a significant enrichment for developmental and cell cycle regulatory pathway genes (Figure 14B).

Interestingly, among the most significantly repressed were the hoxa cluster genes. These genes are critical to early brainstem development and their repression in H3.3K27M cells suggest there may also be inhibition of differentiation caused by the mutation. We performed qPCR validation for the hoxa cluster and found that a large subset of the cluster was repressed in H3.3K27M cells (Figure 15). The H3.3K27M-induced repression of Hoxa genes could explain the inhibition of differentiation we initially observed in our differentiation experiments. Indeed, many of the genes that
were repressed by expression of the H3.3K27M mutation are involved in development, cell fate and cell cycle regulation.

![Image of The Hox Genes bar graph]

**Figure 15:** Repression of Hoxa cluster in H3.3K27M mouse tumors

Importantly, the critical tumor suppressor locus *Cdkn2a* was also among the newly repressed genes. This indicates that H3.3K27M leads to altered PRC2 gene repression, which causes a unique gene expression pattern reflecting the loss of H3K27me3. Importantly, we also observed decreased repression of PRC2 target genes.
similar to what has been observed in patient data. The newly repressed genes could be playing an active role in the H3.3K27M-induced increased proliferation.

4.2 p16/Ink4a repression by H3.3K27M

The CDKN2A locus is among the most commonly altered genes in all cancers including pediatric high-grade gliomas localized in the cortex\textsuperscript{10}. Interestingly, DIPG sequencing studies have shown that the CDKN2A locus is largely devoid of genomic alterations impeding its function\textsuperscript{29}. Therefore, finding this gene as a potential target for aberrant EZH2 inhibition by expression of H3.3K27M mutation suggests epigenetic repression mechanisms are acting to reduce the tumor suppressor functions of Cdkn2a. This could indicate that therapies targeting the downstream Cdkn2a pathway may prove to be efficacious treatments for DIPG and warrant further study. The cdkn2a locus harbors both the p16/ink4a and p19/Arf alternate transcripts, which act on distinct tumor suppressor pathways. p19/Arf acts as by inhibiting Mdm2-mediated proteolytic degradation of p53 resulting in the activation of apoptosis. Whereas p16/ink4a acts as an inhibitor of CDK4/6 phosphorylation of RB, leading to cell cycle arrest or senescence. We utilized qPCR to analyze both alternate transcripts in order to distinguish if one or both were repressed by H3.3K27M expression in our tumor derived neurosphere cells. Interestingly, our results showed that there was specific p16 downregulation, (p=0.0001) with no significant change in p19 transcripts in the H3.3K27M cultured tumor cells compared to the H3.3WT controls (Figure 16A, B). To test whether this repression is a
direct result of H3.3K27M expression alone, we collected brainstem progenitors from neonatal nestin-tva mice, and used RCAS virus to infection with either 1.) H3.3K27M, 2.) H3.3WT, or 3.) empty vector virus. After 2 passages (21 days) we collected the infected progenitor cells, extracted their RNA, and quantified p16 and p19 expression levels by qPCR.

Figure 16: H3.3K27M specifically represses p16 alternative transcript of Cdkn2a
These results confirmed that expression of H3.3K27M alone was able to significantly induce a reduction of p16 expression compared to the H3.3WT and empty vector controls (Figure 16C,D p= 0.007). Thus, these results suggest the H3.3K27M mutation specifically represses p16 without altering p19 expression levels, potentially contributing to the accelerated cell cycle progression and increased proliferation seen in our mutant tumor cells.

5. p16 promoter H3K27me3 indirectly tied to repression

Recent data from DIPG patients has shown that along with the global reduction in H3K27me3, there are also focal loci along the chromatin, which gain this repressive mark and have reduced expression. Work from Chan et al. indicates that the Cdkn2a locus is potentially a site gaining H3K27me3 and subsequent repression. We therefore hypothesized that this may be the mechanism leading to p16 repression in our model. Here we investigate whether H3.3K27M causes changes in p16 promoter H3K27me3 through ChIP experiments targeting H3K27me3 localization to the Cdkn2a locus and whether this alters gene expression.

5.1 Increased p16 promoter H3K27me3

As the CDKN2A locus is a known PRC2 target we analyzed H3K27me3 levels at the p16 promoter as a possible H3.3K27M-induced silencing mechanism. Previous work with H3.3K27M DIPG samples show strong evidence suggesting that despite a global loss of H3K27me3, local genomic regions can harbor gain of H3K27me3 at critical
tumor suppressor genes, including the CDKN2A locus\textsuperscript{46}. We performed ChIP-qPCR on our in vitro GFP-purified tumor lines using antibodies specific for H3K27me3, EZH2, and GFP. We used the Hoxa11 promoter region as a positive control for H3K27me3 ChIP, as this gene is expressed during development but heavily silenced by PRC2 after early embryogenesis\textsuperscript{120}. Additionally, we used the actin promoter region as a negative control for our H3K27me3 ChIP as this gene is highly active and should show little silencing. Results from our controls confirmed that our ChIP experiment was well optimized as we found high levels of H3K27me3 at the Hoxa11 promoter (~100% of input), and very low levels within the Actin promoter (Figure 17).

![Figure 17: ChIP-qPCR positive and negative controls for H3K27me3](image)

We next designed primers to examine the H3K27me3 levels throughout different locations along the p16 and and p19 genes. We chose to design primers at the p16 promoter region, p16 exon 1\textalpha, p16 exon 2, as well as within the p19 promoter region.
Our results for H3K27me3 ChIP showed that H3.3K27M tumor cells had significantly increased H3K27me3 levels at the p16 promoter (p=0.046, Figure 18A). We found no difference in the levels of H3K27me3 at the p19 promoter between H3.3K27M and H3.3WT cells, elucidating why we observe no change in expression of this Cdkn2a alternative transcript (Figure 18B). Additionally, we performed ChIP analysis for EZH2 levels at the p16 promoter and found that while it was present at the locus, there was no significant change in localization between H3.3K27M mutants and H3.3WT controls (Figure 18C). While there was a trend for higher H3K27me3 at exon 1α, and exon 2, there was no significant differences between H3.3K27M and H3.3WT cells at these regions, however promoter methylation is most important for gene silencing (Figure 18D,E).

![Figure 18: H3K27me3 levels throughout the Cdkn2a locus](image-url)
We next performed ChIP analysis using a GFP antibody in order to determine whether there were differences in the chromatin integration between the two GFP-tagged H3.3K27M and H3.3WT exogenous histone constructs. Both the H3.3K27M and H3.3WT exogenous histones integrated into the p16 promoter region at equal levels suggesting that the difference observed for H3K27me3 is not a result of reduced occupancy of the mutant oncohistone at this locus (Figure 19). Therefore the increase in p16 promoter H3K27me3 in the H3.3K27M mutant cells is not a result of the absence of mutant histone within the region.

![Figure 19: Exogenous histone construct integration into p16 promoter](image-url)
5.2 EZH2 promoter methylation is a secondary repressor

To test whether the increased p16 promoter H3K27me3 in H3.3K27M expressing cells is responsible for gene repression, we treated our in vitro murine tumor lines with three potent EZH2 inhibitors, GSK343, GSK126, and EPZ6438. These inhibitors have been tested in clinical trials and are potent inhibitors of EZH2 activity, effectively reducing the levels of H3K27me3. Inhibition of EZH2 is an effective treatment strategy against several types of lymphomas with activating EZH2 mutations. Therefore we hypothesized that since we see aberrant EZH2 activity localized to the p16 promoter, EZH2 inhibitions could remove the promoter H3K27me3 and inhibit tumor cell proliferation.

To test this, we first incubated our high-grade H3.3K27M and H3.3WT expressing tumor cells for 72hr with GK3126, GSK343, and EPZ6438 to determine the IC50 of these EZH2 inhibitors. Surprisingly, no IC50 was reached for any of the EZH2 inhibitors tested, however there was a significant increase in proliferation (p= 0.02) in H3.3K27M cells compared to H3.3WT at 1µM of GSK126, potentially an artifact as increasing concentrations were not significantly changed. Interestingly there was a significant reduction for GSK343 in H3.3K27M cells at 10µM (p= 0.013), however this is a concentration believed to be a non-specific response to epigenetic inhibitors (Figure 20). Together these results indicate that EZH2 inhibition is not a feasible treatment for DIPG.
We next tested whether the EZH2 inhibitors could significantly reduce H3K27me3 levels. For this we incubated H3.3K27M and H3.3WT high-grade tumor cells with GSK343 at 1µM for 7 days. We next used histone extraction followed by western blot to analyze the levels of H3k27me3, and total H3 proteins. We found that after 7 days of incubation there was almost no detectable H3K27me3 signal by western blot (Figure 21A,B).

Figure 21: Pharmacological inhibition of EZH2 globally reduces H3K27me3 in H3.3K27M and WT cells
High-grade tumor-derived cells were incubated with 1µM GSK343 for 7 days prior to A.) Hisone extraction followed by western blot for H3K27me3 and total H3 and B.) Quantification of western using ImageJ software (* <0.05)

In order to ensure that the EZH2 inhibitors were reducing the levels of H3K27me3 at the p16 promoter of both H3.3K27M and H3.3WT high-grade tumor cells, we treated both with 1µM for 7 days and collected the cells for ChIP-qPCR analysis. Our results revealed that EZH2 inhibition led to a specific and significant reduction in promoter H3K27me3 at the p16 promoter of both H3.3K27M and H3.3WT tumor cells (Figure 22A). Additionally, we examined whether the EZH2 inhibitors were changing the incorporation of our GFP-tagged histone constructs at the p16 promoter by performing ChIP-qPCR using an anti-GFP antibody and the same p16 promoter primers. We found that there was no change in histone localization in response to the EZH2 inhibitor treatment (Figure 22B). Therefore we can conclude that the EZH2 inhibitors reduced H3K27me3 at global levels as well as their occupancy at the p16 promoter region without significantly altering the incorporation of H3.3 exogenous constructs.
Figure 22: Pharmacological inhibition of EZH2 significantly reduces p16 promoter H3K27me3

High-grade tumor-derived cells were incubated with GSK343 for 7 days before collected for ChIP-qPCR analysis of A.) H3K27me3 and B.) GFP-tagged histones at the p16 promoter region (* <0.05, ** <0.001)

Finally we investigated whether removal of the promoter H3K27me3 by EZH2 inhibitor treatment led to a subsequent increase in the activating H3K4me3 mark at the p16 promoter by ChIP-qPCR analysis. We used the same 7day 1µM treatment regimen and performed ChIP-qPCR using an anti-H3K4me3 antibody along with primers for the p16 promoter region. Interestingly we found that there was a slight, yet significant increase in H3K4me3 only in the H3.3K27M mutant cells after EZH2 inhibitor treatment (Figure 23). The increased H3K4me3 suggests there could be increased p16 gene expression with EZH2 inhibitor treatment.
Figure 23: H3K4me3 levels in response to EZH2 inhibition

ChIP-qPCR was performed using an anti-H3K4me3 antibody in high-grade tumor derived cells treated for 7 days with EZH2 inhibitor GSK126.

Therefore, we next examined both p16 and p19 expression levels in response to EZH2 pharmacological inhibition using our specific p16 and p19 qPCR primers. Surprisingly, while the EZH2 inhibitors reduced p16 H3K27me3 to near background levels with a corresponding slight increase in H3K4me3, there was no resultant rescue of p16 expression and no change in proliferation by cell counting (Figure 24A,B). This data supports recent findings by another group which has shown evidence that EZH2 inhibitors are not effective against patient DIPG cell lines in vitro\textsuperscript{124}. Taken together, these data suggest that there are additional regulators involved in the H3.3K27M-induced inhibition of p16.
Figure 24: No rescue of p16 with EZH2 inhibition

A.) High-grade tumor derived cells were collected after 7 days of EZH2 inhibition and assessed for p16 transcript rescue. B.) During EZH2 inhibition, wells were counted at 0, 3, and 6 days after drug treatment.
6. DNA methylation represses p16

DNA methylation is a well established epigenetic silencing mechanism known for its role in X chromosome inactivation. This DNA methylation occurs at CpG nucleotides within the chromatin and can also occur at CpG islands, which are long repeat stretches of about 300 CpG nucleotides, that are highly enriched in many gene promoters including that of Cdkn2a. Importantly, the H3K2me3 mark is strongly linked to DNA methylation and has been shown to act as a recruiter of DNA methylation under certain conditions including cancer. Therefore, we hypothesize that DNA methylation may play a role in the H3.3K27M-induced repression of p16 expression. We analyze DNA methylation by pyrosequencing in response to H3.3K27M expression. Additionally we test the affect of DNA methyltransferase inhibitors to examine whether this can be a therapeutic target by rescuing p16 expression and reducing proliferation.

6.1 H3.3K27M increases DNA methylation at p16 promoter

Studies have shown that PRC2 components, including EZH2, interact with and recruit DNA methyltransferases to sites enriched in H3K27me3 for additional gene silencing. DNA methylome analyses of pediatric high-grade glioma patient samples with and without H3.3K27M mutations have shown a global DNA hypomethylation pattern in H3.3K27M positive tumors mirroring the H3K27me3 global reduction. This suggests these two repressive epigenetic systems may work in parallel to repress genes in the context of H3.3K27M mutations. We hypothesize that DNA methylation is
increased and overlaps the H3K27me3 within the CpG island of the p16 promoter. This could explain why pharmacological inhibition of EZH2 and removal of p16 promoter H3K27me3 had no effect on p16 expression or cell proliferation.

Figure 25: Schematic of DNA methylation primer locations

To examine this we utilized a bisulfite followed by pyrosequencing technique in order to quantify the percent of methylated DNA within the p16 promoter region overlapping the increased H3K27me3 (Figure 25). We found 6 out of the 8 H3.3K27M and only 3 out of the 9 H3.3WT tumor-derived neurosphere lines exhibited p16 promoter DNA methylation at higher than 20%, a cutoff indicating promoter silencing or activation. However, while this shows a trend for higher promoter DNA methylation in the H3.3K27M mutants, this was not enough to show a significant difference between the groups (Figure 26A).
Additionally, we examined whether there is increased localization of DNMT1 to the p16 promoter by ChIP-qPCR. However, we found no apparent difference in localization of DNMT1 between H3.3K27M and H3.3WT expressing tumor cells (Figure 26B). We next tested whether pharmacological inhibition of EZH2 had an effect on the DNA methylation levels at the p16 promoter using EZH2 inhibitor treatment. However our analysis shows that there is no change in DNA methylation levels at the p16 promoter in response the H3K27me3 depletion in both H3.3K27M and H3.3WT tumor cells (Figure 26C).

Figure 26: DNA methylation profile of high-grade tumor cells expressing H3K27M

A.) Pyrosequencing analysis showing percent DNA methylation at the p16 promoter CpG island. B.) ChIP-qPCR analysis of DNMT1 localization to the p16 promoter. C.) Pyrosequencing analysis of DNA methylation at the p16 promoter after a 7day EZH2 inhibition treatment.
6.2 DNA methylation regulates p16 expression in H3.3K27M expressing cells

To assess whether the promoter DNA methylation is responsible for regulating p16 expression in H3.3K7M mutant tumor cells we utilized pharmacological inhibition of DNMT1. We treated our in vitro murine tumor cells with the potent DNA methyltransferase inhibitor decitabine and examined its effect on p16 promoter DNA methylation levels, cell proliferation, and p16 expression levels. To examine whether DNMT1 inhibition is able to reduce p16 promoter DNA methylation levels, we used a treatment regimen of 1µM decitabine for 72 hours on tumor neurosphere cells. Our results showed that the DNMT1 inhibitor treatment significantly reduced p16 promoter DNA methylation in only the H3.3K27M lines, although we observed a trend for decreased promoter DNA methylation in the H3.3WT lines this was not a significant reduction (Figure 27A).

We next examined the impact of DNMT1 inhibition on p16 expression by qPCR using the same 1µM for 72hrs treatment regimen. Strikingly, this treatment significantly rescued p16 expression in the H3.3K27M lines to levels similar to the H3.3WT samples. Interestingly, the H3.3WT lines did not show a significant response to DNA methylation inhibition in regard to p16 expression (Figure 27B). Additionally, decitabine treatment exhibited a potent anti-proliferative effect as measured by IC50 BrdU analysis independent of the H3.3K27M mutation (Figure 27C). All together, these data show that
promoter DNA methylation is the critical repressive mark regulating p16 expression in H3.3K27M mutant cells.

Figure 27: Inhibition of DNA methylation in H3.3K27M expressing tumor-derived cells

A.) Pyrosequencing analysis of p16 promoter DNA methylation with 1µM decitabine treatment for 72hrs. B.) qPCR analysis of p16 expression after 1µM decitabine or vehicle treatment for 72hrs. C.) IC50 analysis of decitabine on tumor neurospheres using BrdU incorporation.
7. **p16 repression is central to H3.3K27M aggressiveness**

To further elucidate the role of H3.3K27M-induced p16 repression in the increased proliferation and decreased survival seen in H3.3K27M expressing tumor mice, we used a Cdkn2a knockout murine tumor model. In this system the entire Cdkn2a locus is floxed, therefore both p16 and p19 alternative transcripts are knocked out, which represents a caveat to this study. However, by genetically knocking out the Cdkn2a locus, here we compare the proliferation and survival of high-grade tumor mice expressing H3.3K27M or H3.3WT both *in vitro* and *in vivo*.

**7.1 p16 is central to increased proliferation**

We utilized a cdkn2a floxed, nestin-tva mouse in our RCAS murine tumor model in order to determine whether reduced p16 expression is a key constituent of H3.3K27M-mediated increased tumor aggression. We produced tumors in nestin tv-a; cdkn2a^fl/fl^ mice by injection of RCAS viruses containing PDGFB, p53shRNA, and either the H3.3WT-GFP or H3.3K27M-GFP RCAS virus-producing cells along with RCAS-Cre or empty vector in order to analyze the impact of cdkn2a loss in our tumor models. A small, yet important caveat to this model is that the p19/Arf alternate transcript is also deleted in these mice. However, we do not expect the additional deletion of p19 has any major impact on our findings since the p19 tumor suppressor functions as a p53 stabilizer and we are using knock down of p53 in this model. We used power analysis to
determine that 20 mice per group would be sufficient in order to determine the impact of H3.3K27M in this model.

To analyze how Cdkn2a knockout affects survival in H3.3K27M vs H3.3WT high-grade tumor mice we compared 4 injection groups in nestin-tva; Cdkn2a- floxed mice as follows 1.) PDGFB + H3.3K27M + p53shRNA + empty vector, 2.) PDGFB + H3.3WT + p53shRNA + empty vector, 3.) PDGFB + H3.3K27M + p53shRNA + Cre, and 4.) PDGFB + H3.3WT + p53shRNA + Cre. The H3.3K27M; Cdkn2a<sup>−/−</sup> group showed a significantly reduced survival time with a median survival of 37 days as compared to the H3.3WT; Cdkn2a<sup>+/−</sup> controls which had a median survival of 46 days in the Cdkn2a WT groups, similar to our results in the PDGFB alone and p53 KO high-grade tumor models (Figure 28 p=0.016).

![Figure 28: Survival of H3.3K27M vs H3.3WT tumor mice in response to Cdkn2a genetic deletion](image-url)
Importantly, the Cdkn2a knockout groups exhibited the most aggressive tumor development. Both the H3.3WT and H3.3K27M expressing groups had similar tumor latency, with a median survival of 30 and 32 days, respectively and had significantly shorter survival than the cdkn2a WT groups (H3.3K27M vs H3.3K27M; Cdkn2a+/− p=0.0007, H3.3K27M vs H3.3WT; Cdkn2a+/− p=0.0001, and H3.3WT vs H3.3WT; Cdkn2a+/− p=0.0001). Notably, the cdkn2a knockout groups showed that the H3.3K27M-induced survival difference was completely abolished. This provides strong evidence suggesting p16/ink4a repression by H3.3K27M is a critical contributor to the enhanced tumor aggressiveness in mutant expressing mice.

7.2 Targeting the p16 pathway

The p16 tumor suppressor is an important regulator of cell cycle entry by inhibiting cyclin dependent kinases 4 and 6 (CDK4/6) from phosphorylating RB protein leading to inhibition of S-phase entry\textsuperscript{125}. Cancers with reduction or loss of p16 activity have been shown to benefit from CDK4/6 inhibitor therapies. As we find that H3.3K27M-induces epigenetic repression of p16, we hypothesize that targeting the p16 pathway with CDK4/6 inhibition may be an effective therapy for DIPG. Therefore, we utilized palbociclib, a potent and specific CDK4/6 inhibitor, which is FDA-approved for the treatment of breast cancer, to specifically target the pathway\textsuperscript{126,127}. Additionally, our previous work has shown that palbociclib significantly increases survival in a murine
brainstem glioma model induced with PDGF-B in Cdkn2a knockout mice, however we have not yet analyzed the drug in H3.3K27M expressing cells\textsuperscript{10c}.

We first analyzed the IC50 for mutant and WT tumor cells by BrdU analysis. Our results showed that the H3.3K27M expressing cells have a significant sensitivity to palbociclib treatment in comparison to the H3.3WT control cells with an IC50 of 0.18µM, and 1.5µM, respectively (Figure 29A). We next performed cell cycle analysis of H3.3K27M and H3.3WT tumor cells treated with 2.5µM palbociclib for 48 hours. We found that there was no effect of the drug on cell cycle distribution in H3.3WT cells, however the percentage of H3.3K27M cells undergoing S-phase entry was significantly reduced with the treatment (Figure 29B).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure29.png}
\caption{Analysis of CDK4/6 inhibitor palbociclib treatment on H3.3K27M vs H3.3WT tumor cells}
\end{figure}

A.) IC50 analysis of palbociclib on H3.3WT and H3.3K27M murine tumor neurosphere cells using BrdU incorporation (n=3, p=0.002). B.) Cell cycle analysis of
murine tumor neurospheres after a 48hr incubation with 1μM palbociclib (n=3, *= p< 0.05, **=p< 0.001)
8. Discussion

Advances in DIPG therapy have been impeded largely by the lack of efficacy of adjuvant therapies and accurate pre-clinical models. The discovery of prevalent H3.3K27M mutations in DIPG has been critical in unraveling the biology and potential tumor-driving mechanisms of this disease. The past decade has seen a vast accumulation of genetic and epigenetic information on DIPG, bringing the field closer to breakthroughs for treatment. Understanding how the hallmark H3.3K27M mutation contributes to DIPG development and progression is key in developing efficacious therapies. In this study we use genetic mouse modeling to establish that H3.3K27M expression alone is not sufficient for gliomagenesis although it does promote gene and protein expression alternations leading to a unique signature. We go on to show that H3.3K27M directly accelerates gliomagenesis by cooperating with PDGF signaling. Furthermore, our work demonstrates that the H3.3K27M leads to the epigenetic repression of p16 and that although H3K27me3 deposition is increased at the promoter, DNA methylation is responsible for the repression. Finally, we pharmacologically target the p16 tumor suppressor pathway and show that this is a potential efficacious therapeutic avenue for DIPG patients exhibiting H3.3K27M mutation.

Expression of H3.3K27M alone is not sufficient for tumorigenesis
Since H3.3K27M mutations are prevalent in almost 80% of DIPG, it is important to determine whether it alone is sufficient to induce tumorigenesis. Our in vitro studies found that H.3.K27M expression led to decreased neuronal maturation in cultured mouse brainstem progenitors. Inhibition of differentiation suggests that this could be a mechanism in which the mutant histone could be contributing to the enhancement of gliomagenesis. Indeed, our RNA-Seq analysis of our high-grade tumor models revealed that many of the significantly altered genes, specifically those that were de-repressed in response to the mutant, were involved in differentiation and mature neuronal functions. This is not surprising, as PRC2 target genes are known to be regulators of cell fate decisions. Additionally, there is evidence in the literature using DIPG cell models supporting an inhibition of differentiation to mature neurons in H3.3K27M mutants. Interestingly, while we observe this effect in vitro, the in vivo expression of the histone mutation in our RCAS model was not sufficient to induce gliomas under the conditions we used.

Although we did see expression of our viral constructs in the target brainstem progenitors, there are a number of reasons that H3.3K27M expression did not cause glioma formation. Firstly, it may be true that expression of the histone mutation alone is not sufficient for gliomagenesis. This is the most likely cause as H3.3K27M mutations are commonly found in patients with other mutations, usually p53 inactivating alterations or PDGF amplifications. This is supported by our findings combining p53
loss with H3.3K27M, showing that while this was still not able to produce gliomas, there were ectopic proliferating clusters. Nevertheless, our ChIP-Seq and proteomics analysis of neuronal progenitors expressing the mutation alone revealed that H3.3K27M alters normal patterns leading to unique epigenetic and proteomic signatures. Therefore, perhaps H3.3K27M is capable of tumor initiation under specific circumstances, such as in utero.

As PRC2 is critical during development, it is possible that H3.3K27M mutation may promote gliomagenesis at an earlier time point than we examined such as embryonically. It is very difficult to retrieve this type of information from human patients, however future studies could examine this possibility. Additionally, expression levels of the histone mutation may have to be a specific level to induce tumors alone. As our system overexpresses these constructs, we may have to use the endogenous expression of H3.3 in order to see the true effects of the mutation.

H3.3K27M cooperates with PDGF signaling and p53 loss to accelerate tumorigenesis

Previous patient studies have implicated that the presence of H3.3K27M mutation correlates with increased malignancy and decreased survival\textsuperscript{34}. Patient data also indicate that H3.3 mutations often associate with PDGFRA amplifications and TP53 alterations\textsuperscript{35,36}. These data suggest that there is a relationship between the tumor driving
mechanisms of these alterations. However, until now only one study, by Funato et al., has provided evidence linking these genetic hits to increased malignancy. A previous RCAS model targeting Pax3-expressing cells, with PDGFB, p53 knockout and H3.3K27M expression showed no significant impact on survival relative to PDGFB and p53 knockout alone\textsuperscript{128}. However, by targeting nestin-expressing cells in the models used in this work, we provide the first direct \textit{in vivo} evidence of H3.3K27M-driven acceleration of gliomagenesis by cooperating with PDGF signaling in a genetic mouse model. The different results observed between the Pax3-Tv-a and Nestin-Tv-a models highlights inherent differences between these progenitor populations and the cell-type specific pro-tumorigenic effects of H3.3K27M.

Our previous work revealed that H3.3K27M expression combined with p53 loss induced ectopic proliferating clusters, but was not sufficient to induce tumors\textsuperscript{37}. Our work here suggests the H3.3K27M mutation requires PDGF signaling as a mitogen to exert its pro-tumorigenic effects. Based on previous studies of patient samples indicating that the mutation is invariably associated with obligate partners, including alterations in p53 and growth factor pathways, it is not surprising that H3.3K27M cannot promote or initiate glioma as a single factor\textsuperscript{129}. It remains to be seen whether the histone mutations (either H3.1 or H3.3) induce similar effects in the context of other genetic alterations such as \textit{PPM1D} mutations, \textit{ACVR1} mutations, or PI3K pathway alterations, all of which are found in DIPG\textsuperscript{33,130}. 
Epigenetic mechanisms may play a role in H3.3K27M-induced p16 repression

RNA-Seq analysis of our murine tumors revealed a substantial number of differentially expressed genes, including most notably a subset of repressed genes in H3.3K27M tumors. Past works have identified novel H3K27me3 peaks that arise with H3.3K27M expression and correlate with gene silencing. Importantly the work by Chan et al. indirectly suggested that H3.3K27M expression induced specific p16 repression by gain of H3K27me3 using normal neural stem cells as controls. Our work adds to this by revealing that the p16 suppression is a direct result of H3.3K27M expression both in vivo, in a controlled mouse model that targets a likely cell-of-origin for DIPG, and in vitro. Importantly, expression of H3.3K27M as a single factor is capable of specific p16 repression in ex-vivo neuronal progenitors.

While CDKN2A locus alteration is common to many cancers including adult high-grade gliomas and pediatric high-grade gliomas in the cerebral cortex, it is very rarely observed in DIPG. However, our work reveals that expression of H3.3K27M may be recruiting epigenetic regulators to repress p16. We find that H3.3K27M induces a focal increase in p16 promoter H3K27me3, a canonically repressive epigenetic mark. However, EZH2 inhibitors, although effective at reducing the H3K27me3 gain at the p16 promoter in H3.3K27M cells, do not rescue p16 expression or inhibit proliferation. This is in agreement with a recent report in which EZH2 inhibitors were shown to be
ineffective against human DIPG lines in vitro suggesting additional repressive systems are involved\textsuperscript{124}.

Continued p16 repression after eliminating the promoter H3K27me3 gain suggests the presence of additional regulation acting dependent or independently of the repressive histone mark. H3K27me3 is known to recruit DNA methyltransferases (DNMTs) to drive de novo DNA methylation in certain cancers, and the Cdkn2a locus can be regulated by both H3K27me3 and DNA methylation of promoter CpG islands\textsuperscript{92,118,132}. The relationship between these epigenetic regulators is complex but known to be interdependent\textsuperscript{85,91,93,133}. This remains true in the context of H3.3K27M as tumors from pediatric glioma patients harboring these mutations exhibit global reduction of H3K27me3 and a DNA hypomethylation profile\textsuperscript{48}. Therefore it is possible that, in the context of H3.3K27M expression, regions of aberrant gains in H3K27me3 may also harbor increased DNA methylation providing an additional mechanism of repression.

Supportive of this hypothesis we find that regions of DNA methylation overlap with regions of increased H3K27me3 within the p16 promoter. Critically, rescue of p16 expression is dependent on inhibition of DNA methylation. However, the direct mechanism as to how expression of H3.3K27M mutation leads to increased DNA methylation regulating p16 expression is yet to be determined and is highly complicated by other factors. Indeed, recent evidence shows that hypoxia can induce loss of TET enzyme activity, which catalyzes DNA demethylation, and therefore drives DNA
hypermethylation in tumors\textsuperscript{134}. However, several works have shown that PRC2 binding to H3.3K27M stalls the complex, which may divert unbound PRC2 complexes to regions with low H3K27M occupancy\textsuperscript{46,47,135}. Although we did not observe increased localization of DNMT1 to the p16 promoter, which is responsible for the maintenance of DNA methylation during replication, DNMT3A or DNMT3B, which are the only other mammalian DNMTs and perform \textit{de novo} methylation of unmethylated, and can methylate hemimethylated DNA, may be responsible\textsuperscript{136}. Thus, further investigation is required to determine whether the H3.3K27M-induced increase in H3K27me3 acts as a recruiting platform for DNA methylation or whether other unknown factors are involved is needed.

\textbf{H3.3K27M-induced repression of p16 drives accelerated gliomagenesis}

 Preferential repression of p16 is known to enhance mitogenic responsiveness and promote tumor progression\textsuperscript{132}. Therefore the H3.3K27M-induced p16 repression and subsequent acceleration of cycling cells may be a key component in the enhanced gliomagenesis downstream of the mutation. Indeed, H3.3K27M expression imparts increased sensitivity to the CDK4/6 inhibitor palbociclib compared to H3.3WT cells. Our data indicate that palbociclib, currently in clinical trials for the 10-30\% of patients with genetic aberrations of the RB pathway, may be effective for a much larger percentage of DIPG patients than originally presumed.
Essential to this work, genetic knockout of p16 eliminated the H3.3K27M-induced survival difference between mutant and control mice, suggesting that repression of this gene is key in H3.3K27M-enhanced gliomagenesis. Together these findings support our working hypothesis whereby PDGF signaling and localized H3.3K27M-induced repression of p16 cooperate to accelerate tumor cell growth and increase malignancy in DIPG models (Figure 30). This work highlights that rescue of p16 activity through inhibitors of DNA methylation, CDK4/6, or a combination strategy is a promising avenue for DIPG therapy that may impart additional efficacy to radiation alone.
**Figure 30: Schematic of working hypothesis of H3.3K27M induced p16 repression**

i.) The known function of H3.3K27M mutation in gene de-repression by inhibiting PRC2 function through EZH2 inhibition, potentially through sequestering. ii.) Hypothesized mechanism of H3.3K27M-induced novel gene repression involving DNMT recruitment by PRC2 and subsequent p16 promoter DNA methylation leading to reduced inhibition of CDK4/6 phosphorylation and increased cell cycle activation.

### 8.1 Future directions

Our investigation of the impact of H3.3K27M mutation on brainstem gliomagenesis offers an important contribution to understanding the molecular mechanisms altered by this mutation and highlights potential avenues to the advancement of DIPG therapy. However, our work represents only the establishment of
research on H3.3K27M and its effect on global epigenetics and tumor enhancement. Notably, we did not fully prove whether the increased promoter H3K27me3 as a consequence of H3.3K27M mutation is needed for repression of p16 and whether the DNA methylation regulated repression is seen at the other downregulated genes from our RNA-Seq study. From our work here, I hypothesize that PRC2 is stalled on H3.3K27M residues within the p16 promoter and this leads to direct binding and recruitment of DNMTs to the locus for further DNA methylation gene silencing. However, further work should examine whether there is direct binding of these complexes at the p16 promoter. Additionally, although we did not see a therapeutic benefit from EZH2 inhibition in vitro, performing an in vivo analysis of this therapeutic avenue would be beneficial. Although the neurosphere culture conditions we chose have been shown to best retain the in vivo gene expression of the original tumor, it is still not perfect. The brainstem microenvironment is difficult to mimic in culture conditions and this could drastically change the ability of EZH2 inhibition to exert an anti-proliferative effect. In parallel, it is also important to test the DNA methylation inhibitors in vivo to determine whether the strong anti-proliferative effects we observed can be repeated under more realistic biological conditions.

While we reveal that H3.3K27M induces localized repression of p16, and that rescuing its expression is a good therapeutic option, it is critical to understand why we see localization of the histone mutations and epigenetic silencing mechanisms to these
specific genetic loci. Current knowledge of H3.3K27M shows that this missense mutation causes a gain-of-function to inhibit PRC2 activity, therefore is it possible that other H3.3-associated proteins are affected? The H3.3-specific histone chaperone HIRA is know for its function in depositing H3.3 at developmental loci in embryonic cells, therefore it’s specificity may be altered by the change induced by K27M. A future study using ChIP-Seq to compare H3.3WT against H3.3K27M deposition chromatin could be beneficial to our understanding of how K27M affects H3.3 localization.

Additionally, studies have also shown that PRC2 can be recruited to specific loci by long non-coding RNAs (lncRNAs), which bind to PRC2 and guide the complex. The H3.3K27M-induced loss of H3K27me3 throughout the epigenome may lead to increased expression of certain lncRNAs that recruit PRC2 to novel loci for repression. Importantly, the ANRIL lncRNA has been identified in humans as a potential regulator of the CDKN2B and CDKN2A loci through recruiting PRC2 silencing. This lncRNA could be directly involved in the p16 silencing we observe in H3.3K27M mutants. An important future study should examine this possibility. Currently there is little known about PRC2 recruitment, however it is clear that H3.3K27M is inducing changes in PRC2 localization and further insight into the mechanisms involved in this would benefit the field of DIPG epigenetics and could help in the development of efficacious therapeutics.
Materials and methods

In vivo mouse model experiments

All animals were maintained in accordance with the Duke Animal Care and Use Committee under the approved protocol (A162-16-07). The following mice were used: Nestin Tv-a; p53<sup>fl/fl</sup> (C57BL/6J background) as previously described<sup>106</sup>. Nestin Tv-a; CDKN2A<sup>fl/fl</sup> mice (C57BL/6J background) were obtained by crossing nestin tv-a mice with <i>cdkn2a<sup>fl/fl</sup></i> mice, provided by Ron Depinho. Mice were monitored for tumor growth by weight loss and neurological symptoms.

Cell lines and culture

Murine tumors were isolated and enzymatically digested as previously described<sup>106</sup>. GFP-positive single cells were sorted by FACS and cultured in Neurocult media with proliferation supplement (Stem Cell Technologies) as neurospheres. Cells were split using Accutase (Innovative Cell Technologies) and all experiments were performed using primary (non-thawed) lines at low passage (under passage 10). For BrdU analysis, single cells were seeded into 96-well plates in biological and experimental triplicate, cultured for 72hrs, and then assessed for BrdU incorporation using the Cell Proliferation ELISA Kit (Roche) per the manufacturer’s recommendation. For drug treatments of both murine and human DIPG lines, single cells were seeded into
96-well plates in biological and experimental triplicate and treated with drugs or vehicle (0.1% DMSO) the following day. Primary pediatric human glioma cell lines, SF8628, and DIPG007 (HSJD-DIPG-007), contain H3.3K27M mutations, SU-DIPG-IV contains H3.1K27M, and all lines were obtained from Dr. Rintaro Hashizume at Northwestern University, Dr. Michelle Monje at Stanford University, and Dr. Angel Montero Carcaboso at Hospital Sant Joan de Déu Barcelona, Spain, in accord with institutionally approved protocol at each institution and the cell culture models have been previously described. SF8628 cells derived from surgical biopsy are maintained as an exponentially growing monolayer in complete medium consisting of Dulbecco’s modified Eagle’s medium (DMEM, GIBCO 11965, Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum with penicillin-streptomycin and plasmocin. SU-DIPG IV cell culture derived from DIPG autopsy tissue are grown as tumor neurospheres in Tumor Stem Media (TSM) consisting of DMEM/F12 (Invitrogen, Carlsbad, CA), Neurobasal(-A) (Invitrogen, Carlsbad, CA), B27(-A) (Invitrogen, Carlsbad, CA), human-bFGF (20ng/ml) (Shenandoah Biotech, Warwick, PA), human-EGF (20ng/ml) (Shenandoah, Biotech, Warwick, PA), human PDGF-AB (20ng/ml) (Shenandoah, Biotech, Warwick, PA) and heparin (10ng/ml). DIPG007 (HSJD-DIPG-007) cells are derived from the autopsy are maintained as an exponentially growing monolayer in TSM media supplemented with 5% fetal bovine serum. Human cell histone mutational status was determined using Sanger sequencing for the H3F3A and
HI1ST3B genes. Human cell cultures were validated by DNA fingerprinting using short tandem repeat (STR) analysis and checked for mycoplasma contamination. Human lines were cultured for 2-3 passages (2 weeks) following thaw for experiments. Other experimental details are as described in the text. Inhibitors used were GSK343, GSK126, EPZ-6438, Decitabine, azacitidine, and palbociclib (Selleck). No mycoplasma testing regimen was performed on murine cell lines as they are early passage tumor-derived cells.

**Differentiation assays**

Isolated murine progenitors cells grown as neurospheres were dissociated into single cells before seeding at 100,000 cells per well onto 24 well plates with matrigel coated coverslips. Glass coverslips were incubated for 2hrs at room temperature with Matrigel at a concentration of 85ug/mL. After incubation the matrigel solution was removed and either 0.5mL of neurosphere media (for undifferentiated controls) or DMEM with 10% FBS (for differentiation) was added to each well. After seeding cells, they were incubated for 7 days before beginning IF analysis for differentiation markers.

**Generation of murine brainstem gliomas**

All animal studies were performed in accordance with the Duke University Animal Care and Use Committee and Guide for the Care and Use of Laboratory Animals (protocol # A214-13-08). The RCAS/TVA system was used to generate murine
brainstem gliomas. DF1 virus producing cells were purchased from ATCC, cultured in DMEM (ATCC) supplemented with 10% FBS, 2mM L-glutamine, 100 units/mL penicillin and 100µg/mL streptomycin, and incubated at 39°C and 5% CO₂. Cells were transfected with RCAS plasmids (RCAS-PDGF-B, RCAS-Cre, RCAS-H3.3WT, RCAS-H3.3K27M, RCAS-p53shRNA) using X-TremeGENE 9 (Roche) per the manufacturer’s instructions. 1µL (10⁵ cells) of DF1 cells (ATCC Manassas, VA) expressing equal ratios of RCAS virus-producing cells were injected intracranially into the brainstem of postnatal day 3-4 nestin tv-a (Ntv-a) p53fl/fl mice to generate brainstem gliomas as previously described. Mice were euthanized with CO₂ upon appearance of symptoms of brain tumor development (weight loss, lethargy, head tilt, or hydrocephalus) in accordance with Duke University IACUC protocol. Brain tissue from these animals was extracted and either fixed in 10% neutral buffered formalin and paraffin embedded or used to generate tumor cell lines.

In vitro infection of brainstem progenitors with RCAS viruses

Normal brainstem progenitors from nestin-tv-a mice were isolated and infected with concentrated RCAS-H3.3K27M-HA, RCAS-H3.3WT-HA, or RCASY (empty vector) viruses as previously described and grown under neurosphere conditions.

Tumor grading
Tumor samples fixed in 10% formalin for 24hrs were embedded in paraffin by the Duke Pathology Core and cut into 5µM sections using a Leica RM2235 microtome. H&E staining was performed using standard protocols. Tumor grading was performed by a blinded neuropathologist (RM).

**Immunohistochemistry analysis**

Formalin fixed brains were paraffin embedded by Duke Pathology Core Services. Sections were cut 5µm thick using a Leica RM2235 Microtome. Immunohistochemistry was performed using an automated processor (Discovery XT, Ventana Medical Systems, Inc.) Antibodies used are provided in the supplemental material. Quantification was performed using Metamorph software. We analyzed 10 different high-powered fields (40x), and quantified total nuclear area of positive staining to total nuclear area.

**Immunofluorescence studies**

Tumor-bearing mice were injected with EdU (10mg/kg) 4hrs prior to sacrifice, brains were fixed in 10% formalin, paraffin embedded and cut into 5µM sections. Slides were deparaffinized in xylenes and rehydrated with decreasing ethanol solutions. EdU staining was performed following the protocol for Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen #C10339). Quantification was performed using Metamorph
software. We analyzed 10 different high-powered fields (40x), and quantified total EdU positive staining to total nuclear area (DAPI stained).

**Western blots**

Western blots on histone-extracted proteins were performed as previously described \(^{37}\). Antibodies used are provided in the supplementary material. Quantification of band intensities was performed using Image J software.

**MRI Tumor volume analysis**

T2-weighted scans of the brain were acquired using a cryogenic coil (Cryoprobe, Bruker Biospin, Billerica, MA) and the following parameters: repetition time TR=1.5s, echo time TE=22ms, bandwidth 75kHz, flip angle 90, matrix: 160x160, 100 slices, field-of-view 1.6x1.6x1.0cm. Tumor volumes were obtained from manual segmentation.

**RNA-seq analysis**

Read-pairs were aligned to the mouse genome (mm9) by Expression Analysis. The number of read-pairs per gene was determined based upon the NCBIM37 gene annotation from the ENSEMBL database \(^{146}\). Genes that did not have at least 10 read-pairs in any single library were excluded from further analysis. Normalization and
differential expression analysis was carried out using the DESeq bioconductor package (default parameters) \(^{147}\).

**Reverse transcription, Real-time Quantitative PCR (qRT-PCR) analysis**

Total RNA was isolated using RNeasy kit (QIAGEN) per the manufactures protocol. cDNA was synthesized from total mRNA using Superscript II and OligodT primers (Invitrogen). qRT-PCR TaqMan primers were used for murine p16, and murine p19 and sequences are provided in the supplementary material. Relative gene expression levels were generated using the ΔΔCt method \(^{148}\).

### Table 3. TaqMan qPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16/Ink4a</td>
<td>CGGTCGTACCCCGATTCAG</td>
<td>GCACCGTAGTTGACGAAGAG</td>
<td>AACGTGCCCATCATCA</td>
</tr>
<tr>
<td>p19/Arf</td>
<td>TGAGGCTAGAGAGGATTGAGAAG</td>
<td>GTGAACGTTGAGCCATCATCAT</td>
<td>ACCTGGTCCAGGTTC</td>
</tr>
</tbody>
</table>

**Flow Cytometry**

Cell cycle analysis by flow cytometry was performed by fixing cells in 70% ethanol on ice for 15mins. Cells were washed, stained with propidium iodide labeling solution (BD), and transferred to FACS tubes. Cell cycle analysis was performed by the Duke Cancer Center Flow Cytometry Core.
Chromatin Immunoprecipitation followed by quantitative PCR (ChIP-qPCR)

Tumor neurospheres were dissociated using Accutase (Innovative Cell Technologies) and counted using the Scepter automated cell counter (Millipore). Five million cells were fixed in 1% formaldehyde for 7mins at room temperature, quenched with the addition of 125mM glycine for 5mins and washed with 1xPBS before storing at -80°C. Cells were resuspended in 500μL lysis buffer (10mM HEPES, 0.5% NP-40, 1.5mM MgCl₂, 10mM KCl) with protease inhibitors and incubated on ice for 10mins. After centrifugation at 5,000RPM for 5mins the nuclear pellet was lysed in 500μL nuclear lysis buffer (50mM Tris, 1%SDS, 10mM EDTA) with protease inhibitors for 15mins. Extracts were sonicated, cleared by centrifugation, and diluted to 1 million cells per mL in dilution buffer (0.01%SDS, 1.1% Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris). Antibody incubation (4μg) was performed overnight. ChIP antibodies used are located in the supplemental material. 40μL of 50% Protein A/G beads (GE Healthcare) were added to lysates and rotated at 4C for 3hrs. Protein bound beads were sequentially washed with wash I (20mM Tris, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1%SDS), wash II (20mM Tris, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), wash III (10mM Tris, 250mM LiCl, 1mM EDTA, 1% NP-40, 1% deoxycholate), then washed twice with TE. DNA was eluted with 1% SDS, 0.1M NaHCO₃ and reverse cross-linked by incubation at 65C with RNase overnight. Eluants were then treated with proteinase K.
and DNA was purified using QIAGEN PCR purification kit. DNA amplification was performed by qPCR as described above.

<table>
<thead>
<tr>
<th>Gene/ location</th>
<th>Forward (5’→ 3’)</th>
<th>Reverse (5’→ 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16/Ink4a promoter</td>
<td>CTGTTTCAACGCCCAGCTCTC</td>
<td>GATGGAGCCCGGACTACAAGAAG</td>
</tr>
<tr>
<td>p16 exon 1α</td>
<td>CGAACCACGGAGAGCCATC</td>
<td>ACACCCCTTGCTACCTGAA</td>
</tr>
<tr>
<td>p16 exon 2</td>
<td>TCACTACGGTGCAAGATTGC</td>
<td>ATCGCACGATGTCTTGATGC</td>
</tr>
<tr>
<td>p19/Arf promoter</td>
<td>CGTACCGCTAAGGGTTCAA</td>
<td>TTTCGCTTCCGTAACTTTC</td>
</tr>
<tr>
<td>Hoxa11 promoter</td>
<td>AGCCCAATGATGAGTTTGA</td>
<td>GAAGGGAGGCTGGAGAAATC</td>
</tr>
<tr>
<td>β-actin promoter</td>
<td>GAATGTGGCTGCAAAGAGTCTAC</td>
<td>CTTCGCTCTCTCGTGCTGCTAGTA</td>
</tr>
</tbody>
</table>

**Table 4. ChIP-qPCR primers**

**ChIP-Seq**

ChIP-Seq service was performed by Zymo Research Corporation. Briefly, cells were cross-linked with formaldehyde at 1% final concentration for 7 min at room temperature, and chromatin prepared using the Zymo-Spin ChIP kit (Zymo Research Corp., Irvine, CA) following manufacturer’s instructions. Sonication was performed at high power setting for 40 cycles (30 s on, 30 s off) using an Bioruptor Plus (Diagenode Inc., Denville, NJ), yielding fragment size range of 200-700 bp. ChIP assays were performed in triplicate for each sample using 10 µg of chromatin and 5 µg of anti-H3K27me3 (Millipore, 07-449; Lot 2455635) and anti-H3K4me3 (Millipore, 07-473; lot 2500603). IgG (Millipore, PP64B; Lot 2565474) was included as a negative control.
ChIP DNA was purified using ChIP DNA Clean and Concentrator (Zymo Research, Irvine, CA) and the relative abundance of control regions GAPDH and HOXA11 in either H3K4me3 or H3K27m3 immunoprecipitated DNA and input DNA respectively were quantified by quantitative real-time PCR with sequence-specific primers. DNA libraries were prepared and sequenced on a HiSeq sequencer (Illumina, San Diego, CA). H3K4me3 and K3K27me3 ChIP-Seq reads were aligned to mouse reference genome mm10 by Bowtie with at most 2 mismatches. Reads that appeared more than twice at the same position on the same strand were discarded to remove PCR duplication. BIGWIG files were generated from the coverage for visualization purposes. MACS2 was used to identify H3K4me3 and H3K27me3 peaks at q-value cutoff 0.01 using broad peak setting. In addition, sample peaks for H3.3 WT, empty vector, and K27m samples were compared to each other and those overlapping by more than 50% were considered “common” to both samples, otherwise the two peaks would be considered “unique”.

Proteomics studies

Sample preparation:

The Duke Proteomics Core Facility (DPCF) received nine cell pellets. The samples were resuspended in 200 µL of 50 mM ammonium bicarbonate with 0.2% ALS-I. Samples were subjected to four rounds of probe sonication for 5s each with an energy
setting of 30% Samples were then centrifuged at 12,000 g at 4C for 5 minutes. Protein concentrations were determined by Bradford assay on the supernatant in duplicate. Concentrations ranged from 1.17 µg/µL to 5.78 µg/µL with total protein ranging from 227 µg to 1120 µg. 175.5 µg of total protein was removed from each sample and 50 mM ammonium bicarbonate with 0.2% ALS-I was added to normalize all samples to 1.17 µg/µL protein. Samples were supplemented with 5.3 pmol of undigested casein (to give 30 fmol of casein per µg of protein) as an intact protein as an internal standard for phosphopeptide enrichment. Samples were reduced with 10 mM DTT at 80C for 15 min and then alkylated at 25 mM iodoacetamide at room temperature for 30 min. Trypsin was added to a 1:50 ratio (enzyme to total protein) and allowed to proceed for 18 hr at 37C. Samples were then acidified with 0.2% TFA (pH 2.5) for 2 hr at 60C to hydrolyze ALS-I surfactant. Following centrifugation, supernatants were split such that 170 µg was processed through the phosphopeptide enrichment workflow and 5.5 µg was reserved for unbiased LC-MS/MS. All samples were then frozen and then lyophilized to dryness.

TiO2 enrichment:

Lyophilized peptides for phosphorylation enrichment were resuspended in 65 µL of 1M glycolic acid in 80% acetonitrile/1% TFA. Phosphopeptide enrichments were performed on 170 µg of total peptide using 10 µL GL Bioscience TiO2 spin tips as per a standard protocol. Eluted phosphopeptides were then lyophilized to dryness and resuspended in 70 µL of 0.15% TFA. Enriched peptides were subjected to a C18 cleanup
using the C18 stage tips. Samples were lyophilized to dryness and resuspended in 12 µL of 10 mM citric acid in 0.1%TFA/2% acetonitrile with 12.5 fmol/uL yeast alcohol dehydrogenase. To create a “QC pool” sample to assess analytical reproducibility, 3 µL of each sample was removed and pooled.

Quantitative analysis of Phosphorylation Enriched and Unbiased Samples:

All phosphorylation enriched samples were run first followed by unbiased samples. Samples were randomized in their run order and QC samples were run periodically throughout the acquisition window. Quantitative LC/MS/MS was performed on 4 µL of each phosphopeptide enriched sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Thermo QExactive Plus high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 300 mm Å~ 180 mm trapping column (5 _l/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.7 µm Acquity BEH130 C18 75 mm Å~ 250 mm column (Waters Corp.) using a 5-min hold at 3% acetonitrile with 0.1% formic acid and then a 90-min gradient of 3 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55C. Data collection on the QExactive Plus mass spectrometer was performed in a data-dependent acquisition (DDA) mode of acquisition with a r=70,000 (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of 1e6 ions followed by 10 MS/MS scans at r-17,500 (@ m/z 200)
at a target AGC value of 5e4 ions. A 20s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each sample injection was approximately 2-hours.

Unbiased samples were resuspended in 22 µL of 1% TFA/2% acetonitrile with 50 fmol/µL yeast alcohol dehydrogenase. To create a “QC pool” sample to assess analytical reproducibility, 3 µL of each sample was removed and pooled. Quantitative LC/MS/MS was performed on 1 µL of each unbiased sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Thermo QExactive Plus high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 300 mm Å~ 180 mm trapping column (5 l/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.7 µm Acquity BEH130 C18 75 mm Å~ 250 mm column (Waters Corp.) using a 5-min hold at 3% acetonitrile with 0.1% formic acid and then a 90-min gradient of 5 to 40% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55C. Data collection on the QExactive Plus mass spectrometer was performed in a data-dependent acquisition (DDA) mode of acquisition with a r=70,000 (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of 1e6 ions followed by 10 MS/MS scans at r-17,500 (@ m/z 200) at a target AGC value of 5e4 ions. A 20s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each sample injection was
approximately 2-hours. Following the 26 LC-MS/MS analyses, data were imported into Rosetta Elucidator v3.3 (Rosetta Biosoftware, Inc), and all LC-MS/MS runs were aligned based on the accurate mass and retention time of detected ions (“features”) which contained MS/MS spectra using PeakTeller algorithm (Elucidator). In the phosphorylation dataset, all LC-MS data was intensity-scaled based on only those peptides which were identified as being phosphorylated. In the unbiased dataset, a mean normalization of the high confidence identified peptide features excluding the highest and lowest 10% of the identified signals (i.e. a robust mean normalization) was employed. The relative peptide abundance was calculated based on area-under-the-curve (AUC) of aligned features across all runs. The phosphorylation and unbiased datasets had 71,222 and 71,131 quantified isotope (peptide) groups, respectively. Additionally, 388,281 and 427,341 MS/MS spectra were acquired for peptide sequencing by database searching for the phosphorylation and unbiased datasets, respectively. These MS/MS data were searched against an NCBI_RefSeq_Mouse (Dec 2012, 26,696 forward entries) database containing bovine casein, bovine serum albumin, and yeast alcohol dehydrogenase sequences. This database was appended with a reversed-sequence “decoy” database of the same set of forward entries for false positive rate determination. Database searching was performed within Mascot Server v2.5 (Matrix Science) and phosphorylated data were annotated at a Mascot ion score of 20 resulted in peptide false discovery rates of 0.64%. Unbiased data were run through the
PeptideTeller algorithm set to a peptide false discovery rate of <1.0%. Searching allowed variable modification on S, T, and Y (+80 Da, phosphorylation dataset only) and M oxidation (+16 Da).

Pyrosequencing

Primers for CDKN2a pyrosequencing assays were designed using PSQ assay design software version 1.0.6 (Qiagen). The primer sequences and PCR conditions are provided in the supplementary material. Seven microliters PCR products were used for pyrosequencing following the protocol from the manufacturer (Qiagen).

    Pyrosequencing primers:

    F1: TGTTAAAGGGTGATTAGGTATGGG

    R1: ACCCCTAAAAAACACTACTATCCC

    S1: GATTAGGTATGGGGAG

Sequence to Analyze: GGGTGTTAGY GTGGGTAGTA GGYGGGGGTG GTTYGATTTT
TTAGYGTTGT TITAAYGTIT AGTTTTTTT TTGAATTTTGTATT

Statistical Analysis

Statistical analysis was performed using Graphpad Prism (Version 6.0). All survival curves were analyzed by Log-Rank (Mantel- Cox) test. IHC and IF analyses were performed using the Mann-Whitney test. IC50 and growth curve determinations
and significance calculations were found by nonlinear regression analysis. All other assays were performed using either paired or unpaired standard t-test.

**Antibodies**

**Immunohistochemistry and IF:**

- Rabbit anti-H3K27me3 (Cell Signaling 9733S), Rabbit anti-GFP (Abcam ab290),
- Rabbit anti-Olig2 (Millipore AB9610), Rabbit anti-phospho H3 (Millipore 04-1093),
- Mouse anti-Rat nestin (BD Pharmigen 556309), PCNA

**Western Blots:**

- Mouse specific: Goat anti-Actin (Santa Cruz sc-1616), Rabbit anti- H3 (Abcam ab1791), Rabbit anti- H3K27me3 (Cell Signaling 9733S), Rabbit anti-GFP (Abcam ab290)
- Human specific: Mouse anti- human p16/ink4a (BD Pharmingen 554097)

**Chromatin Immunoprecipitation:**

- Mouse anti- H3K27me3 (Abcam ab6002), Rabbit anti-GFP (Abcam ab290), Rabbit anti- H3K4me3 (Abcam ab8580), rabbit anti-DNMT1 (Abcam ab13537)
Appendix A

DIPG BBB permeability and H3.3K27M

Brainstem pediatric high-grade gliomas (pHGGs) are diagnosed based on MRI findings and a combination of neurological symptoms\textsuperscript{6}. Given the critical importance of in vivo imaging in the diagnosis of pHGGs, several research groups have investigated the feasibility of using different MR pulse sequences as a potential prognostic marker for disease progression\textsuperscript{149-152}. As pediatric brainstem HGGs show limited enhancement in contrast-enhanced T1-weighted MRI, and due to the high-frequency of H3.3K27M mutations in this disease group, we hypothesized that both tumor location and H3.3K27M status may influence BBB permeability. In this study, we used dynamic contrast-enhanced (DCE) MRI to evaluate the permeability of the blood–brain-barrier of a genetically engineered mouse model of pHGG as a function of location (cerebral cortex vs. brainstem) and H3.3K27M mutation status (mutant vs. wild-type).

*High-grade tumor mouse model presents similar imaging characteristics to human DIPG*

We induced brainstem and cortical gliomas driven by PDGF-B, p53 loss and either H3.3K27M or H3.3 wild-type as previously described\textsuperscript{110}. All tumors included in this work were histologically confirmed to be high-grade gliomas defined by the presence of microvascular proliferation and/or necrosis. Pseudopalisading necrosis and vascular proliferation can be seen in a representative H&E section (Figure 31). The MR
image features of the murine tumors reflect the clinical equivalents observed in children. Gliomas appear hyperintense on \(T2\)-weighted images and hypo/iso-intense on \(T1\)-weighted images (Figure 32).

Figure 31: H&E images comparing high-grade glioma models in brainstem vs cortex

H&E images were taken at 40x magnification from high-grade glioma groups in the a.) cortex with H3.3WT, or b.) cortex with H3.3K27M, and compared to high-grade gliomas in the c.) brainstem with H3.3WT, or d.) brainstem with H3.3K27M.

The pre-injection \(T1\) values in the tumor volume typically were \((\mu \pm \sigma) . 1.54 \pm 0.21\) s. Figure 32(b) depicts the functional \(K^{\text{trans}}\) map at the same axial location as the slice in figure 31a. Graphs of the temporal evolution of the concentration of the contrast agent
at two pixels (arrowheads in figure 32b) with distinct permeability parameters are given in figure 32d. Figure 33 demonstrates the heterogeneity of BBB permeability in a typical cortical glioma.

**Figure 32: Representative MR images of cortical glioma**

a.) $T_2$-weighted and c.) hypo/iso- intense on $T_1$-weighted MRI. B.) $K^{\text{trans}}$ map at same axial location. d.) Concentration of contrast agent as function of time at locations indicated by arrowheads in b.)
Figure 33: Orthogonal views of $K_{\text{trans}}$ map

a.) Axial, b.) saggital, and c.) Coronal planes. d.) $K_{\text{trans}}$ histogram over tumor volume in representative glioma

**Brainstem glioma BBB permeability is reduced compared to cortical tumors, but not regulated by H3.3K27M**

The $K_{\text{trans}}$ maps from a representative animal in each genotype group are compared in figure 34. The outline of the tumor volume determined from the corresponding $T2$-weighted image is overlaid for reference. Figure 34 (a, b) shows a
high-grade glioma with the H3.3WT or H3.3K27M histone mutation originating in the cortex; Fig. 34 (c, d) presents the respective brainstem glioma.

![Figure 34](image)

**Figure 34: Representative $K^{trans}$ maps from each genotype group**

High-grade glioma maps from a.) Cortex with H3.3WT, b.) Cortex with H3.3K27M, c.) brainstem with H3.3WT, and d.) brainstem with H3.3K27M. e.) group comparison of mean $K^{trans}$

Notice the inhomogeneous spatial distribution and distinctly higher $K^{trans}$ values in the cortical tumors. This heterogeneity is characterized in Fig. 34(e, f) depicting the
permeability parameter histogram of the separate phenotypes. The distributions are computed by pooling the $K_{\text{trans}}$ histograms from all animals in each specific group. In cortical HGGs the mean BBB permeability was significantly higher than in brainstem HGGs ($p<0.01$); however we found permeability was not regulated by expression of the H3.3K27M mutation ($p=0.78$). The analysis of variance on the square-root transformed distributions confirmed these findings, suggesting BBB permeability is dependent on tumor location and could explain the difficulty in finding an efficacious chemotherapy that can increase survival above the radiation standard of care.

**Cortical high-grade gliomas have increased tumor volume independent of H3.3K27M**

Figure 35(a) plots the time in days since DF1-virus injection until the appearance of glioma symptoms (lethargy, head tilt, and increased head size); the number of days for mice to become symptomatic was not significantly different between the four groups (ANOVA, $p=0.82$).
Figure 35: Comparison of tumor growth vs time dependent on location and genotype
Although on average it took the same number of days for mice to become symptomatic, the volume of the cortical tumors was significantly higher (ANOVA, p<0.01) than the volume of the brainstem tumors. In each location (cortex or brainstem) the difference between the volumes of the gliomas with wild-type or mutated H3.3 histone was not statistically significant (ANOVA, p≈0.51). The observations summarized in Fig. 35(b) imply that the growth rate for tumors in the cortex is significantly higher than for those developing in the brainstem, regardless of H3.3K27M mutation status. Interestingly, we find a significant correlation (p<0.05, ρ=0.43) between HGG volume and mean of $K_{\text{trans}}$ as shown in Fig. 35(c). This finding is suggestive of an invasion mechanism associated with tumor perfusion/permeability.

**Discussion**

Preclinical models are essential in the study of carcinogenesis in a biologically relevant microenvironment\textsuperscript{154}. The advent of genetically engineered mouse models of high-grade gliomas\textsuperscript{155} has provided a unique opportunity for understanding the driving oncogenic events. Using the RCAS genetically engineered mouse modeling system we generated de novo tumors arising from endogenous tissue that closely resemble human DIPG in location, histology, and genetic alterations. The RCAS system enabled for spatiotemporal control of directed oncogene delivery into nestin-expressing neural stem cells of the neonatal mouse brainstem, which previous human studies have provided strong evidence as the cell of origin for DIPG\textsuperscript{74}. This system allowed for real-time
monitoring of the unperturbed, endogenous BBB permeability in both cortical and brainstem tumor locations. Our previous work has shown this is a functional model of the prevalent H3.3K27M mutation found in DIPG, displaying significant global loss of the H3K27me3 mark. We have also used this modeling system in preclinical studies to show that BMS-754807, and PD-0332991, multi-kinase and Cdk4/6 inhibitors respectively, are able to significantly increase survival\textsuperscript{106,110}. Additionally this model is well suited for preclinical studies as it shows similar enhancement by MRI and tumor histology as human DIPG.

The aim of this work was to determine \textit{in vivo} the permeability of the blood-brain barrier in four distinct subgroups of pediatric high-grade gliomas: HGGs with wild-type vs. mutant histone H3.3K27M developing in the brainstem vs. the cortex. K27M mutations are highly prevalent in pediatric brainstem HGG leading to inhibition of the PRC2 complex and a decrease in global H3 trimethylation \textsuperscript{115}. Although these histone mutations occur more commonly in midline tumors they have also been identified in the cortex \textsuperscript{156}. In this study we include the K27M mutation in the cortical HGG group as well in order to control for any effect on BBB permeability attributed to this mutation. Presence of H3K27M mutation has been linked to poor prognosis. Our study provides further insight into DIPG biology, providing further evidence as to why adjuvant chemotherapies have shown no additional efficacy beyond the radiation standard of care. Conventional anatomic MR imaging is the current standard of care for diagnosing
and monitoring tumor progression in pediatric patients with HGG. Using the clinically available MR sequences to date, the parameters of tumor size and invasiveness, metastatic lesions, necrosis, hydrocephalus, or edema have not been able to accurately predict overall survival in these patients. However, the addition of spectroscopy, perfusion, and diffusion MRI has recently been used to identify prognostically distinct subgroups and to correlate to survival time.

In this work we observe that the permeability of the blood-brain barrier in a high-grade glioma mouse model is dependent on the location of the tumor, however expression of the H3.3K27M mutation does not seem to alter permeability. Our study demonstrates that cortical HGG blood vessels have a significantly higher permeability (~150% higher) than those developing in the brainstem. Interestingly, tumor location appears to play a role in tumor growth rate as cortical HGGs had a significantly larger volume than the brainstem HGGs, but symptoms for both conditions appeared at approximately the same time. This difference in tumor volume versus time of symptoms can be attributed to the delicate location of the brainstem HGGs. H3.3K27M mutation status did not lead to a significantly different tumor volume in both brain regions. Mean $K_{\text{trans}}$ was a significant predictor for the volume of gliomas studied in this work.

These results suggest that the reduced permeability of the BBB in brainstem HGGs compared to cortical HGGs is a possible explanation for the lack of response of brainstem HGGs to drug therapy. Given that our experiments controlled for variability
in epigenetic drivers, our findings further illustrate the important role played by the glioma microenvironment in influencing tumor growth and BBB permeability. In summary, DCE-MRI imaging of a genetically engineered mouse model of pediatric HGGs demonstrates that only tumor location plays a significant role in determining BBB permeability. Future work should address whether the extent of the permeability of the BBB influences response to treatment, particularly to therapies (such as bevacizumab) that impact BBB directly\textsuperscript{161}. 
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Biography

Francisco Javier Cordero Jr. was born in Concord, CA on March 7th, 1988. He graduated from the University of California, Davis ('10) with a Bachelors of Science degree in Biochemistry and Molecular Biology. He completed his Doctorate of Philosophy in Pathology with a specialty in Developmental and Stem Cell Biology at Duke University. During his career he has received the Northern California Laborers Scholarship, and Alliance/ Merk Science Hispanic Scholars National Scholarship award while at UC Davis and was awarded a National Research Service Award at Duke University. During graduate school, Francisco was also active in community outreach through the Bouchet Society and The Doing Outreach in Genetics and Genomics organization, as well as tutoring local high school students. He has published articles from his works at UC Davis and Duke University:

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For all manuscripts, * indicates the authors contributed equally to this work