In vivo shRNA screen reveals PRMT5 as a key regulator in glioblastoma

by

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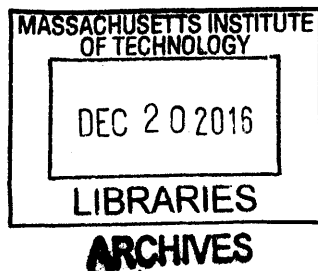
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Abstract

Glioblastoma (GBM), the most prevalent primary brain tumor in adults, remains a largely intractable malignancy that carries an extremely poor prognosis. Large scale genomic analyses have greatly expanded our knowledge of the genomic landscape of GBM. Recently, we have also begun to gain an appreciation for how dysregulation of epigenetic control can contribute to gliomagenesis. To expand our understanding of the roles of epigenetic mediators in GBM, we establish an orthotopic transplantation model and demonstrate its feasibility for *in vivo* shRNA screening. We describe the results of parallel *in vivo* and *in vitro* shRNA screens to uncover epigenetic regulators with oncogenic functions and chemosensitizing potential in GBM. From these screens, the protein arginine methyltransferase PRMT5 emerges as an important mediator of GBM tumor growth in the presence and absence of chemotherapy. We demonstrate that PRMT5 loss impairs cellular fitness *in vitro* and this effect is dependent on its methyltransferase activity. Furthermore, *in vivo* transplantation of both murine and human GBM cells shows that PRMT5 knockdown impairs tumor growth and leads to a survival benefit. Building on these observations, we report the first use of a highly selective PRMT5 inhibitor in extending survival in a GBM xenograft model. We also describe the results of initial *in vitro* and *in vivo* experiments combining PRMT5 genetic depletion or pharmacological inhibition with chemotherapy. To gain a better understanding of the effects of PRMT5 inhibition, we perform gene expression analysis, and find a putative role for PRMT5 in maintaining GBM cell identity. Importantly, we observe that PRMT5 impacts gene expression by regulating RNA splicing to exert specific control over a recently described class of intronic sequences known as detained introns. Together, the results presented here highlight a key role for PRMT5 in GBM and suggest that it represents a viable target for therapeutic intervention in this intractable disease.

Thesis Supervisor: Jacqueline Lees
Title: Professor of Biology

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This thesis represents an intertwined effort, where many paths crossed in direct and indirect ways, both professionally and personally, to help in the formation of the final product. I am grateful to the incredible people who helped guide me through this journey and I would like to take this opportunity to thank them.

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Contents

Chapter 1

Introduction

G LIOBLASTOMA (GBM) is a highly aggressive disease whose prognosis has remained essentially unchanged for the past 50 years. This fact underlies the motivation for my work in this thesis. Although we have succeeded in significantly advancing our understanding of the genetic, and more recently, the epigenetic changes that contribute to GBM, these discoveries have not yet translated into a strong survival improvement for patients. In this thesis, I describe the results of an shRNA screen revealing the protein arginine methyltransferase PRMT5 as a key mediator of tumor growth and therapy response in GBM. To put these results into context, I first provide an introduction on the genetics and epigenetics of cancer. I then describe the PRMT family and focus on established and emerging roles for PRMT5 in cancer. Finally, I provide an overview of GBM and the results of recent large scale studies that have classified this heterogeneous disease into distinct subtypes with different genetic and epigenetic characteristics.

■ 1.1 Cancer Genetics and Epigenetics

■ 1.1.1 Discoveries of genetic and epigenetic alterations in cancer

The twentieth century was marked by a collection of groundbreaking discoveries describing the molecular basis of cancer that are now etched in history. These studies drove the acceptance of cancer as a disease of chromosomal abnormalities [30] and somatic mutations [287]. Although the genetic and epigenetic mechanisms involved in cancer are inextricably intertwined, the contributions of epigenetics to tumorigenesis remained underappreciated for most of the last century [85]. Research focused on the genetic basis of cancer, shaping our understanding of how dysregulation of the delicate

balance between tumor promoting oncogenes and tumor suppressor genes contributes to cancer development. In the middle part of the century, the study of cancer was focused on infectious agents, oncogenic retroviruses that led to the formation of sarcomas in a variety of animals (reviewed in [59]). Scolnick and colleagues [249] first proposed a model in which these viruses transduced a normal cellular gene (later called RAS) into their own genomes, a hypothesis that was proven soon thereafter by Varmus, Bishop and Vogt [261]. Discoveries of how oncogenic sarcoma viruses co-opted cellular genes were complemented by studies of *in vitro* cellular transformation, led by Weinberg and colleagues, showing that human tumor-derived DNA could transform mouse fibroblasts [210]. The combination of these two paths culminated in the identification of HRAS, the first human oncogene [72, 229, 245]. Paving the way for the identification of the first tumor suppressor gene, Knudson proposed the two-hit hypothesis, noting that biallelic genetic alterations were necessary for the development of retinoblastoma tumors [165]. In the following decade, the tumor suppressor gene RB1 was cloned and characterized by Friend, Weinberg and Dryja [94]. These studies, along with many others, began to uncover the complex genetic alterations underlying tumor development, and solidified the view of cancer as a genetic disease.

The field of cancer biology soon experienced a paradigm shift, brought on by the ambitions of Renato Dulbecco [77] and others. They advocated for a systematic discovery of cancer causing genes by sequencing the whole genome, as opposed to focused interrogation of individual genes (reviewed in [104]). The resulting Human Genome Project was launched in 1990 and completed in 2004 [148]. This monumental undertaking also led to the development and widespread implementation of next generation sequencing platforms [136]. These technologies have been instrumental in the success of large scale projects such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). Following the TCGA pilot project in glioblastoma which highlighted that large scale systematic interrogation of the cancer genome was not only possible but also immensely valuable [200], the TCGA has amassed multidimensional data from 33 tumor types and freely shares this data with researchers all around the world. Characterization of mutations, DNA methylation, copy number alterations, gene and protein expression for thousands of tumor samples would have been impossible without the technological developments set in motion by the progressive thinking that led to the human genome project.

The cancer genomics revolution and the technological advancements it spurred have

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changed the pace of discovery in cancer biology, and also accelerated the study of other cellular process, such as epigenetics, metabolism, RNA splicing and their contribution to tumorigenesis [104]. In particular, our exploration of the epigenome has been facilitated by techniques such as high throughput bisulfite sequencing, chromatin immunoprecipitation sequencing (ChIPseq) and chromosome capture experiments [68] that build layers of complexity onto the simple one dimensional view of the genetic sequence. While epigenetics is by no means a new concept, our understanding of the interplay between genetics and epigenetics in rewiring cellular processes that contribute to tumorigenesis has been expanding rapidly and has given rise to novel therapeutics. Epigenetics, a term originally coined by C.H. Waddington in 1942 [292], refers to the impact of chromatin structure on gene function [85, 252]. These heritable changes allow cells that share the same genetic sequences to take on different identities. Studies in the past couple of decades have demonstrated that cancer is initiated and facilitated by a combination of genetic mutations and epigenetic alterations and the contribution of each of these two processes cannot be explored in isolation [252].

■ 1.1.2 DNA hypomethylation and hypermethylation

One of the earliest forms of epigenetic regulation of gene expression to be discovered was mediated by alterations in DNA methylation. In cancer cells, DNA was found to be typically hypomethylated in tumor tissues in comparison to normal tissues [86, 100] and the first oncogene shown to be hypomethylated in human cancer was HRAS [86]. A few years later, reasoning that gene expression is inhibited by DNA methylation, Horsthemke and colleagues showed that the RB1 tumor suppressor gene is hypermethylated in retinoblastoma tumors [115].

We are only now beginning to appreciate the complexity of DNA methylation changes in the cancer genome and how they contribute to tumorigenesis. In general, cancers show both hypermethylation and hypomethylation of certain regions compared to normal tissue controls [83]. In addition, in normal cells, DNA methylation is a heritable, dynamic form of cellular memory (reviewed in [18, 181]). It occurs via the enzymatic actions of DNA methyltransferases (DNMT family) that add methyl groups to the C5 position of cytosine bases. When this modification occurs on CpG rich regions of gene promoters, it results in transcriptional silencing, for example, leading to repression of tumor suppressor genes or genes encoding the components of the DNA repair machinery. For example, in lung and breast cancers, the tumor suppressor p16ink4a is epigeneti-

cally silenced [135, 269] and in colorectal carcinoma the mismatch repair gene MLH1 is hypermethylated [134]. In contrast, genome-wide hypomethylation in cancer is less well understood, but appears to contribute to genomic instability and increased transcription of repeats, transposable elements, as well as oncogenes [18, 83]. DNA methylation changes in cancer have been targeted therapeutically by drugs such 5-azacytidine and decitabine, nucleoside analogs that are incorporated into DNA and form covalent intermediates with DNMTs, inhibiting subsequent rounds of DNA methylation. These drugs are FDA approved for myelodysplasia and acute myeloid leukaemia, respectively [3].

DNA methylation is a dynamic process, involving not only DNMTs but also erasers and readers of the methylation marks. The ten eleven translocation (TET) family of DNA hydroxylases actively reverse DNA methylation, although this process can also occur passively by the inhibition of DNMTs during cell division [181]. Transcriptional repression that occurs as a result of DNA methylation is facilitated in part by methyl-CpG binding proteins (MBDs), which bind methylated DNA motifs and recruit repressive complexes to modify histones and thereby transcription (reviewed in [181]). This begins to illustrate how covalent modifications on DNA and histone modifications work together to modulate chromatin structure and gene expression.

■ 1.1.3 Histone modifications: acetylation and methylation

More than 50 years ago, Allfrey and colleagues proposed that the binding of DNA to histones can be regulated by histone modifications and that acetylation can influence the rate of transcription [5]. The dysregulation of the complex code of histone modifications is now starting to be appreciated as a major contributor to tumorigenesis. The nucleosome is the fundamental unit of chromatin and is composed of DNA wrapped around a protein octamer core, consisting of two each of histones H2A, H2B, H3, and H4. Each histone also has a 'tail" that protrudes from this central core and is rich in lysine and arginine residues (reviewed in [12]). A variety of post translational modifications can be deposited on these amino acids by "writers", removed by "erasers", and interpreted by "readers", which integrate and relay the epigenetic signals (Figure 1.1A, reviewed in [253]). Many of these different players are misregulated in cancer and some have come into focus as therapeutic targets [66]. While methylation and acetylation are the most prominent and well-studied modifications of histone tails, others include ubiquitination, phosphorylation, sumoylation, citrullination, ADP ribosylation and deamination. These modifications work combinatorially to generate a "histone code" that can cause

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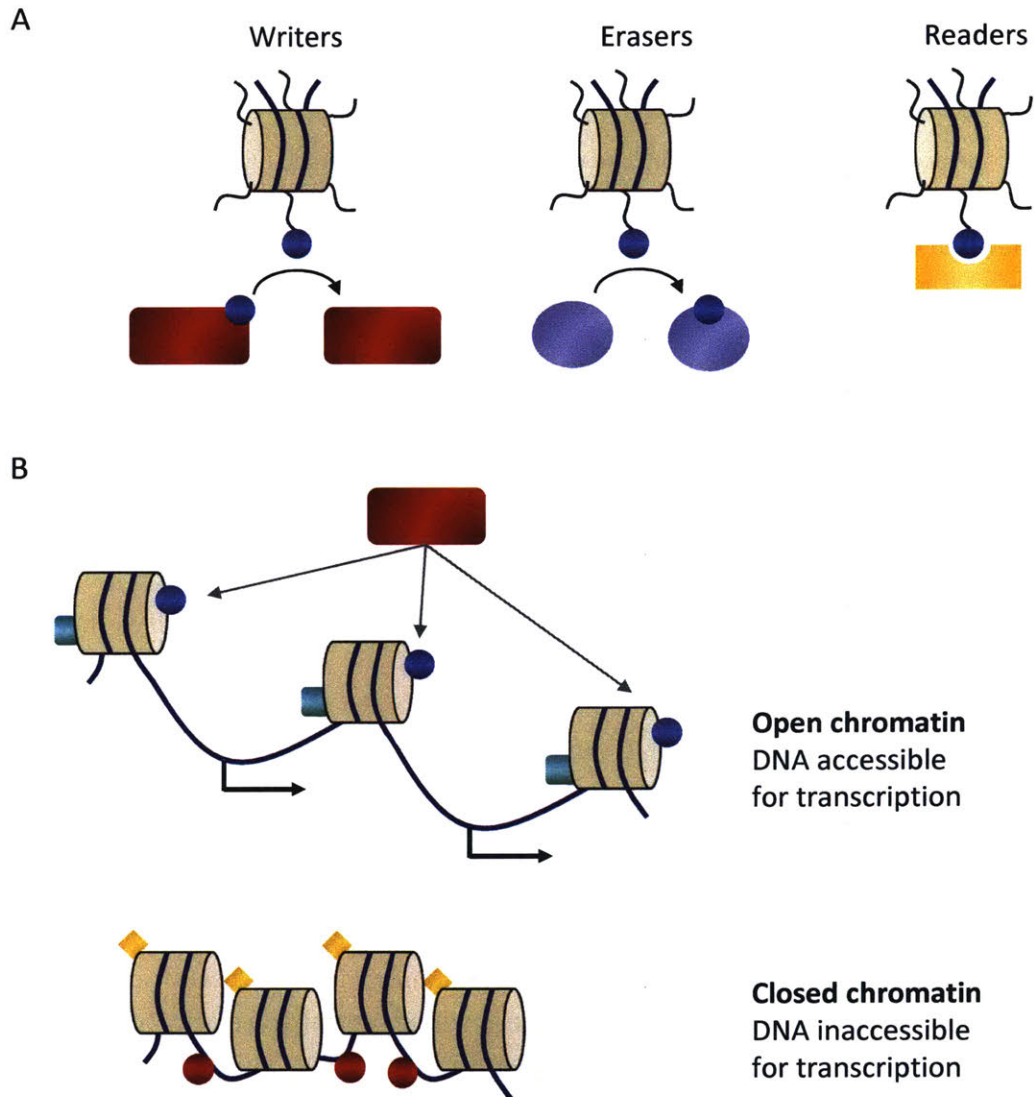
Figure 1.1. Histone modifications. (A) Histone tails are rich in lysine and arginine residues and are subject to posttranslational modifications that are deposited by writers, removed by erasers, and interpreted by readers. (B) The combination of these posttranslational modifications leads to a histone code that dictates chromatin compaction and consequently, gene expression. The DNA in open chromatin is readily accessible for transcription, resulting in increased gene expression, while DNA in closed chromatin is less accessible, leading to decreased gene expression.

increases or decreases in gene expression and chromatin compaction [154] (Figure 1.1B). In addition to regulating gene expression and chromatin condensation, histone modifications also influence DNA repair and DNA replication [168]. Although here I will focus mostly on a subset of these effects, their range and variety are worth noting as part of the intricate web of epigenetic modifications.

Histone acetylation is dictated by the well-orchestrated balance between the actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In broad terms, the addition of an acetyl group to lysine residues results in neutralization of the positive charge and weaker binding between DNA and histones, increasing accessibility of the DNA to transcription factors and RNA polymerase II [66]. In contrast, removal of acetyl groups by HDACs results in a repression of gene expression. Both of these classes of enzymes also modify non-histone targets, and have been found to be important in cancers, through altered expression, translocations resulting in fusion proteins or coding mutations [66]. For example, chromosomal translocations and somatic mutations disrupting the acetyltransferases p300 and CBP, both putative tumor suppressors, have been identified in hematologic and solid tumors [150]. In comparison, HDACs are rarely found to be mutated but more frequently overexpressed in cancers, and have become attractive therapeutic targets. The presumed mechanism of action of HDAC inhibitors is to activate transcription of tumor suppressors. There are currently two broad HDAC inhibitors approved in the clinic for T-cell lymphoma, with other more subtype selective drugs undergoing development [12, 18].

Histone acetyl marks are primarily read by bromodomain containing proteins, many of which also have other functions as histone writers or chromatin remodelers, such as p300 and SMARCA2, underscoring the many levels of epigenetic regulator function. The most prominent example involves the BET family members BRD3 and BRD4, whose genes are involved in recurrent oncogenic chromosomal translocations in acute myeloid leukemia and NUT-midline carcinoma [67, 70, 90, 325]. Several new inhibitors of this family of histone modifiers have shown therapeutic efficacy, at least in part through downregulation of MYC transcription [67, 70, 90, 325].

Histone methylation largely occurs on lysine and arginine residues. These are dynamic marks, deposited by histone lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs) and removed by histone lysine demethylases (KDMs). While the counterbalancing arginine demethylases have thus far remained elusive [28, 74], the reversible nature of other post translational marks suggests that they exist. I

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will discuss PRMTs in greater detail in the next section.

Lysine methylation is deposited by SET motif containing enzymes that are highly specific to certain residues, as opposed to HATs, whose activity is more broad. H3K4 and H3K36 methylation are most often associated with active genes, while methylation of H3K9 and H4K20 is associated with repressed genes [12,17]. Lysines can be mono, di, or tri methylated, and different levels of methylation on the same residue can occur over different genic regions. For example, H3K4me2/3 is found at the promoters of transcriptionally active genes, while H3K4me1 is found at enhancers (reviewed in [17,66]). At times, these marks can coexist, such as in bivalent chromatin domains in embryonic stem cells. In this case, the silencing mark H3K27me3, deposited by EZH2, a Polycomb group protein, can coexist with the active mark H3K4me3, keeping developmental genes in a silent state but poised for activation [21]. Several KMT-encoding genes have been described to be disrupted in cancer. Rearrangements of MLL family members, responsible for H3K4 methylation, are frequently found in myeloid and lymphoid leukemias [322]. Furthermore, EZH2 is overexpressed in breast and prostate cancer [48], as well as mutated in follicular and diffuse large B cell lymphomas [208]. Thus far, small molecule inhibitors of methyltransferases DOT1L and EZH2, and demethylase LSD1 have been put into clinical testing [12]. Readers of methylated lysine marks include members of the chromo, Tudor, and malignant brain tumor (MBT) domains, as well as PHD fingers (reviewed in [11]).

Given the assortment of possible histone modifications, it is not surprising that they can have both positive and negative effects on each other, either by influencing binding or the catalytic activity of the protein complexes that recognize them. This crosstalk adds another dimension of regulation to the histone code [66,168].

■ 1.1.4 Chromatin Remodelers

The basic DNA and histone modifications provide the scaffolds for and determine the binding of large multi-subunit complexes that modulate the three dimensional structure of chromatin. These dynamic units have many interchangeable members and use ATP to facilitate nucleosome remodeling or movement. SWI/SNF complexes are defined into classes containing either SMARCA2 (BRM) or SMARCA4 (BRG1) and are important in coordinating gene expression programs during lineage commitment [308]. Inactivating mutations and tumor suppressor activity have been linked to several members, such

as SNF5 in the majority of rhabdoid tumors, ARID1A in ovarian clear cell carcinoma, and BRG1 in non-small-cell lung cancer (reviewed in [308]). Within the CHD family of chromatin remodelers, certain subclasses contain both ATP-dependent remodelers and histone deacetylases, illustrating how intertwined the roles of epigenetic modifiers are [66, 253].

The integration of DNA and histone modifications with chromatin remodeling results in nucleosome positioning and establishment of boundaries between tightly and loosely packed nucleosomes, which are associated with transcriptional repression or activation, respectively [80]. These transcriptional domains break down during cancer, altering the normal state of transcriptional regulation [17]. Our current understanding of the complexities underlying the abnormalities in cancer requires us to build upon the original hypotheses, for example, by expanding the genetic mechanisms proposed in Knudson's two-hit hypothesis to also include epigenetic pathways for tumor suppressor gene inactivation [155]. Probing into the molecular identities of cancer has helped us to understand that the genome and epigenome are tightly intertwined and one cannot be effectively understood without the other [319].

■ 1.2 The PRMT family of methyltransferases

■ 1.2.1 Overview of PRMT family

Protein arginine methyltransferase (PRMT) family members impact numerous cellular and physiological processes through a diverse array of substrates and have been implicated in pathologies ranging from cardiovascular disease to cancer. The importance of arginine methylation is underscored by its abundance in mammalian tissues, with 0.5% of arginine residues carrying this post-translational modification [105].

PRMT targets include histones, transcription factors, enzymes, RNA binding proteins, scaffolding and adaptor proteins, transport proteins and ion channels. The modifications they impart are important for chromatin remodeling, transcription, DNA repair, RNA splicing, growth factor and hormone signaling, cell cycle, viral life cycle, protein trafficking, tumor suppressor response, extracellular matrix remodeling, and cytokine signaling. PRMTs are critical for normal development and their misregulation has been shown to contribute to cardiovascular and pulmonary diseases, viral pathogenesis, amyotrophic lateral sclerosis, spinal muscular atrophy, and cancer [207].

In mammals, 9 protein arginine methyltransferase (PRMT) family members have been identified thus far [263]. These enzymes catalyze post-translational covalent modifications involving the transfer of methyl groups from donor substrate S-adenosylmethionine (SAM) to a guanidino nitrogen of an arginine residue, forming S-adenosyl-L-homocysteine (SAH) in the process. SAM is generated from methionine as part of the methionine and arginine salvage pathway.

■ 1.2.2 Classes of PRMT enzymes

The PRMT family is divided into three main classes, type I-III, all of which methylate the terminal guanidine nitrogen atom of a protein substrate [19]. All PRMT family members first catalyze the formation of a monomethylarginine (MMA) intermediate, and type I enzymes (PRMT1, 2, 3, 4, 6, 8) then proceed to produce asymmentric dimethylarginine (ADMA), while type II enzymes (PRMT5, 9) produce symmetric dimethylarginine (SDMA) marks (Figure 1.2A). Interestingly, PRMT7 is known to be a type III enzyme, producing MMA without further progressing to dimethylation. Importantly, methylation does not alter the cationic charge of the arginine residue,
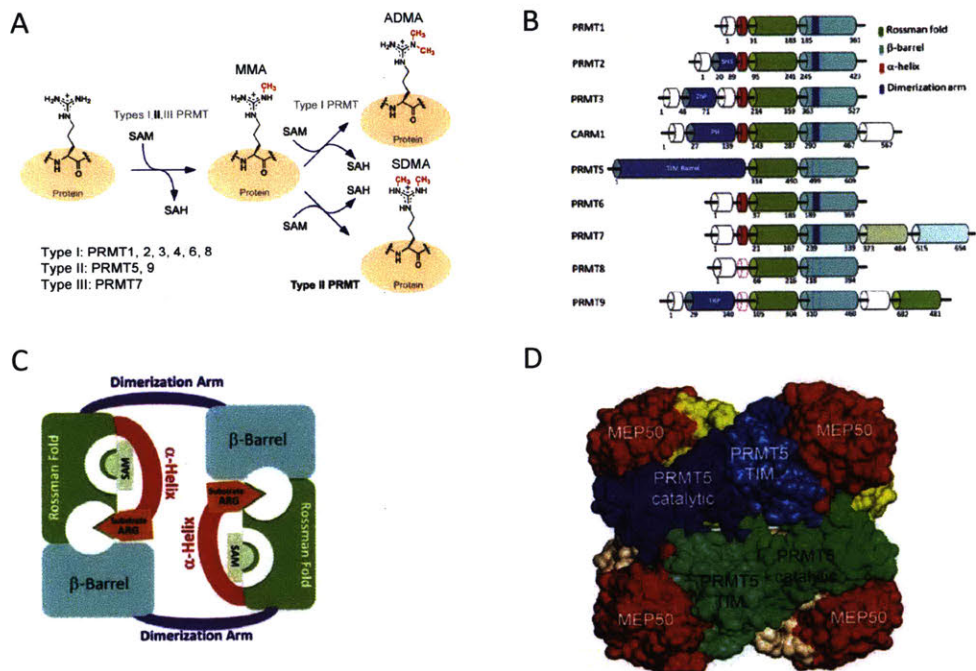
Figure 1.2. Structure and function of the PRMT family of methyltransferases. (A) The protein arginine methyltransferase (PRMT) family consists of three groups: type I, II, and III that catalyze the formation of asymmetric dimetylarginine (ADMA), symmetric dimethylarginine (SDMA), and monomethylarginine (MMA), respectively. Methyl groups are transferred from the cofactor and donor substrate S-adenosylmethionine (SAM) to a guanidino nitrogen of an arginine residue, forming S-adenosyl-L-homocysteine (SAH) in the process (modified from [263]). (B) Structure of PRMT family members, highlighting the variable N terminal region (modified from [247]). (C) Homodimeric arrangement of type I PRMTs (modified from [247]). (D) Four PRMT5 and four MEP 50 molecules form a hetero-octameric complex. PRMT5 monomers-1, -2, -3, and -4 are displayed in green, blue, beige, and yellow, respectively, and MEP50 in red (modified from [10]).

instead increasing its bulkiness and hydrophobicity, and preventing the formation of hydrogen bonds [284].

Previously, certain members of the PRMT family have been thought to function via a distributive (or non-processive) enzymatic mechanism for substrate methylation, such as PRMT5, meaning that the substrate is first monomethylated, and then dissociates from the enzyme [10,297,307]. Only once the concentration of monomethylated substrate exceeds that of unmethylated substrate can dimethylation proceed. Others, such as PRMT1, function via a processive mechanism, whereby a single enzyme-substrate binding event results in two methylation reactions without substrate dissociation [207]. A more recent report, however, indicates that the degree of processivity may be dependent on protein substrate sequence and posttranslational modifications [120].

The three different PRMT catalyzed modifications (MMA, ADMA, SDMA) can have profoundly different biological effects on the same substrate. For example, PRMT1 and PRMT5 antagonize each others activity on E2F1, in that ADMA of E2F1 results in apoptosis, while SDMA favors proliferation [324]. Other studies have demonstrated that H4R3 ADMA via PRMT1 results in transcriptional activation, while PRMT5-mediated SDMA modification of the same residue results in transcriptional repression [207]. These examples illustrate the tight homeostatic control that PRMT family members maintain over their substrates, demonstrating that a shift in activity levels of different PRMTs could have significantly different consequences.

■ 1.2.3 Structural and functional overview of PRMT family members

The PRMT family is characterized by a conserved core composed of a Rossmann fold and a beta-barrel, together forming the methyltransferase catalytic site where SAM and the substrate bind [247]. In this active site, a double-E-loop structure with two highly conserved glutamate residues imparts the negative charges that coordinate the positively charged guanidine nitrogens of the substrate arginine into the correct orientation for catalysis to take place [207]. The N-terminal region is the most variable part of the structure, differing in length, sequence and even protein-protein interaction modules (Figure 1.2B). For example, PRMT2 contains an SH3 domain, PRMT3 has a zinc finger motif, there is a pleckstrin homology (PH) domain in PRMT4 (also known as CARM1), a triosephosphateisomerase (TIM) barrel in PRMT5, an N-myristoylation tag in PRMT8, and a tetratricopeptide (TRP2) domain in PRMT9 (used in formation

of protein complexes). These differences may contribute to the diverse substrate specificities of the different PRMT family members, as well as regulation of their activity and subcellular localization.

PRMT homodimerization appears to be required for methyltranferase activity. Type I PRMTs contain a dimerization arm in the beta-barrel (Figure 1.2C) that allows for the formation of a head-to-tail dimeric arrangement, with each monomer binding its own peptide substrate and cofactor [207, 247]. An alpha-helix helps to stabilize the formation of the active site. PRMT7, the type III enzyme, contains a single polypeptide that folds on itself and mimics the dimeric structure. Meanwhile, PRMT5 has some distinct structural features that contribute to its activity. Unique among PRMT family members, PRMT5 functions as part of several multimeric complexes that invariably contain methylosome protein 50 (MEP50) as a necessary binding partner of PRMT5 [263]. This WD40-repeat containing protein, encoded by the gene WDR77, is non-catalytic but required for full PRMT5 activity [10]. A crystal structure of PRMT5 [10] recently revealed that instead of the alpha-helix characteristic to other family members, the Rossmann fold in this enzyme is linked to a specific TIM barrel via a large liker domain. Two tyrosine side chains in the linker domain facilitate binding to histone substrates [10, 186]. The TIM barrel is responsible for the tetrameric form that is unique to PRMT5, whereby the dimer is first formed by interaction of the TIM barrel of one monomer and the Rossman fold and linker of another. Subsequently, tetramers are formed via the stacking of two dimers. The TIM-barrel facilitates the recruitment of four MEP50 molecules, resulting in the formation of a chromatin binding complex (Figure 1.2D). MEP50 was found to have an allosteric effect on PRMT5 binding of substrate and SAM and this hetero-octomeric complex has significantly higher methyltransferase activity than dimeric PRMT5 [10]. The PRMT5:MEP50 complex is likely to be the core structural unit that participates in many multisubunit protein complexes and facilitates many of the functions of PRMT5 [10].

∎ 1.2.4 PRMT family substrate specificity and regulation

Given the wide range of PRMT targets and the diversity of their functions within the cell, it is important to understand how these enzymes select their substrates and how this interaction is regulated. The majority of PRMTs methylate glycine arginine rich (GAR) motifs, with the exception of CARM1, which only target proline, glycine, and methionine rich (PGM) motifs [20, 153, 313]. Unique in the family, PRMT5 can

methylate both motifs. A recent study has determined that there are more than 2000 human proteins harboring the GAR motif, broadly defined as tri-RGG, di-RGG, tri-RG and di-RG, and many proteins contain more than one of these motifs [277]. Interestingly, these motifs lack any other positively charged amino acids, underscoring the specific importance of arginine. Although PRMTs appear to be basally active, fine-tuning of PRMT activity can occur through several mechanisms, including post-translation modifications, subcellular localization, and interaction with regulatory proteins. I will discuss these mechanisms in more detail in relation to PRMT5 in the next section.

■ 1.2.5 PRMT family tissue expression

In mammals, the predominant type I and type II enzymes are PRMT1 and PRMT5, respectively [19]. PRMT1, 4, and 5 are expressed broadly in various tissues throughout the body. The rest of the family members display more tissue-specific expression, such as PRMT8 which is expressed exclusively in the brain, and PRMT7 which is expressed in the brain, lung, and testes [301]. The importance of these enzymes in mammals is underscored by the severity of their knockout phenotypes. Prmt1 and Prmt5 knockout mice each die early during embryonic development [231, 276]. Carm1 null animals are smaller than their wild type littermates and die shortly after birth [311]. Loss of function of the more tissue specific PRMTs results in less severe phenotypes. Prmt2 null mice are viable and grossly normal [318], Prmt3 knockout mice are largely normal and survive into adulthood [270] and Prmt6 deficient mice are also normal with no overt phenotype [214].

As the main arginine methyltransferase family member that deposits SDMA marks, PRMT5 has come to light as an important orchestrator of proliferation and differentiation, through its interactions with an array of cellular substrates ranging from chromatin modifiers to tyrosine receptor kinases, as discussed below. Unsurprisingly then, dysregulation of PRMT5 activity leads to a wide range of diseases, including cancer (reviewed in [313]). In the rest of this section, I will detail the diverse roles of PRMT5 in the cell, specifically as they relate to our accumulating evidence of its involvement in tumorigenesis.

■ 1.2.6 Discovery of PRMT5

PRMT5 domain organization and structure are highly evolutionarily conserved across eukaryotic species, with all members containing the Rossmann fold, beta-barrel and TIM barrel. *C. elegans* PRMT5, which shares 31% sequence identity with human PRMT5, harbors sequence insertions that prevent its tetramerization [10, 263]. The homolog of human PRMT5 was first identified in *Schizosaccharomyces pombe* as skb1 [108]. The authors were studying Ras effector pathways and how cytoskeletal regulation was mediated by a Rho-associated kinase known as Shk1, the homolog of mammalian p21Cdc42/Rac activated kinases (PAK). Using a two-hybrid screen searching for interactors of Shk1, they identified Skb1 as a positive regulator of Shk1 function, whose overexpression resulted in hyperelongated *S. pombe* cells. At the same time, another PRMT5 homolog, HSL7 (histone synthetic lethal 7), was cloned in *Saccharomyces cerevisiae [195]*. Hints at functional motifs and biochemical activity were only gathered with the identification of human PRMT5, which was first identified as JAK2 binding protein (JB1) in a yeast two-hybrid assay aimed at identifying JAK2 interacting proteins [234]. With no known function for this protein or its homologues, in this elegant work, Pollack and colleagues demonstrated that JB1 had SAM-dependent methyltransferase activity, bound histone and non-histone substrates, and was part of a multi-subunit complex. They characterized its mRNA expression over a wide range of human tissues, and also identified a *Drosophila* homolog of the protein. A couple of years later, Branscombe and colleagues designated this novel gene as PRMT5, the first of the type II PRMTs [32]. They demonstrated its ability to catalyze both MMA and SDMA and found that it was responsible for methylation of myelin basic protein (MBP), as well as Sm ribonucleoproteins, SmD1 and SmD3. Together, these studies began to unravel the complexities of PRMT5 function and spurred the study of the ever expanding list of processes regulated by PRMT5.

■ 1.2.7 PRMT5 is an epigenetic regulator involved in transcriptional regulation via chromatin remodeling

One of the most prominent and well-studied roles of PRMT5 is its modulation of chromatin structure and gene expression by modification of histones. PRMT5 has been shown to symmetrically dimethylate H2AR3, H4R3, H3R8, and H3R2 [263]. Its association with several complexes enables it to have wide-ranging effects on gene regulation,

with different binding partners dictating its target specificity.

In the nucleus, the PRMT5:MEP50 complex binds to COPR5 (cooperator of PRMT5), an adaptor protein that modulates the binding of PRMT5 to chromatin [174]. In U2OS cells, this interaction preferentially promotes methylation of H4R3 at target promoters, resulting in transcriptional repression. PRMT5 has been shown to associate with SWI/SNF complexes of chromatin remodelers that contain either hBRM or BRG ATPases, thereby linking histone dimethylation and chromatin remodeling. These interactions and their outcomes appear to be context dependent, resulting in transcriptional activation or repression [160]. PRMT5 associates with SWI/SNF and catalyzes promoter region methylation of H3R8 and H4R3 and subsequent transcriptional silencing [226]. ChIP analysis in NIH-3T3 cells revealed that targets of these PRMT5-contaning chromatin remodeling complexes include suppressor of tumorigenicity 7 (ST7) and nonmetastatic 23 (NM23), as well as cell cycle regulatory genes. Overexpression of PRMT5 results in reduced expression of these two tumor suppressor genes, and increased cellular transformation [226]. PRMT5 has also been shown to function with chromatin remodeling complexes in a mutually exclusive manner with c-myc [227]. At the cad gene promoter, BRG1-based SWI/SNF complexes associate with the Sin3A/HDAC2 repressor complex together with PRMT5, leading to transcriptional silencing. Sin3A/HDAC2 facilitate deacetylation of H3 and H4, allowing for more efficient PRMT5-mediated dimethylation of the same histones. However, c-myc competes for the repressor-free SWI/SNF complex, and high levels of c-myc result in activation of the target gene [227]. Meanwhile, in other contexts, the interactions between PRMT5 and SWI/SNF complexes result in transcriptional activation. For example, during early myogenic differentiation PRMT5 dimethylates H3R8 at the MYOG promoter [62, 63]. This modification is required for binding and function of BRG1 as part of the SWI/SNF complex to promote chromatin remodeling and subsequent transcriptional activation of MYOG, an essential early gene for skeletal muscle differentiation [62]. In an interesting illustration of the interplay between PRMT family members, PRMT5 is present at late myogenic gene promoters, but dispensable for their activation. Instead, CARM1 facilitates SWI/SNF-dependent late gene expression [62]. Together, these results indicate that the interaction of PRMT5 with SWI/SNF chromatin remodelers can both promote and suppress gene transcription.

PRMT5 has also been shown to interact with the NuRD remodeling complexes, dynamic structures that mediate transcriptional repression by bringing together com-

ponents that bind methylated DNA, deacetylate and methylate histones and ultimately remodel nucleosomes [225]. Methyl CpG binding domain proteins MBD2 and MBD3 were shown to be a part of mutually exclusive NuRD complexes in HeLa and 293T cells, with MBD3 having much lower affinity for methylated CpG islands in DNA [179]. The PRMT5:MEP50 complex only methylates MBD2, owing to the presence of RG rich sequences, which are absent on MBD3. MBD2 recruits PRMT5 to CDKN2A CpG islands, and this interaction was found to be dependent on DNA methylation and correlated with the presence of symmetrically dimethylated histone H4R3 [179]. PRMT5-mediated methylation of MBD2 decreases its affinity for methylated CpG islands, and its ability to recruit HDACs, thereby reducing its activity as a transcriptional repressor [273]. Interestingly, both PRMT1 and PRMT5 methylate MBD2, with indications that PRMT1 mediated methylation may be required for subsequent modification by PRMT5[273]. These studies demonstrate that PRMT5 can modulate the interaction of certain NuRD complexes with DNA and impact their promotion of transcriptional repression.

The newest histone residue found to be modified by PRMT5 is H3R2, whose symmetric dimethylation is important for euchromatin maintenance in human cells. Previous studies had shown that asymmetric dimethylation of H3R2 by PRMT6 prevents binding of WDR5, a component of the MLL complex, thereby inhibiting the deposition of the activating epigenetic mark H3K4me3 [118, 164]. Migliori and colleagues found that symmetric dimethylation of H3R2 by PRMT5 and PRMT7 strongly favored binding of WDR5, and exclusion of Rbbp4 and Rbbp7, part of NuRD/Sin3A and PRC2 repressive complexes [204]. A new report [49] demonstrates that PRMT5-mediated H3R2 monomethylation also recruits WDR5 and promotes H3K4me3 to activate transcription.

PRMT5 has also been shown to interact with other co-repressor complexes, regulating expression of genes that control cell proliferation, differentiation, and cell adhesion [7, 82, 137, 272]. Complex purification and ChIP studies revealed that Ski co-repressor complexes in HeLa cells contained PRMT5, HDAC3, as well as several SMAD proteins, and localized to the SMAD7 promoter, a TGF-β responsive gene [272]. This study revealed that in the absence of TGF-β stimulation, Ski complexes maintain SMAD7 in a repressed state. During germ cell development, PRMT5 associates with the BLIMP1/PRDM1 repressive complex, leading to H2A/H4R3 dimethylation and subsequent inhibition of expression of genes involved in germ cell specification [7, 82].

PRMT5 also associates with AJUBA, a repressive complex that interacts with the EMT transcription factor SNAIL, and is recruited to E-cadherin (CDH1) gene promoter [137]. These examples underscore the variety of repressive complexes that PRMT5 interacts with and the diversity of their cellular roles.

In summary, PRMT5 functions as a transcriptional regulator through histone methylation and interactions with transcriptional co-activator and co-repressor complexes, as well as ATP-dependent chromatin remodelers. The effects of PRMT5 on transcription are therefore highly context dependent.

■ 1.2.8 Mechanisms of regulating PRMT5 activity

PRMT5 activity on histone and non-histone targets must be tightly regulated in order to fine tune its effects and wide range of interacting partners. A diversity of post-translational modifications, protein-protein interactions, and subcellular localization can modulate the activity of PRMT5 in the cell [160, 207, 263]. Although the levels of other histone modifications are regulated via deposition by writers and removed by counterbalancing erasers, a histone arginine demethylase has not been confidently described to date.

Post-translational modifications (PTMs) of either PRMT5 or its binding partner MEP50, can regulate the activity of this methyltransferase complex. For example, an oncogenic gain-of-function mutant of JAK2 aberrantly phosphorylates PRMT5, disrupting its interaction with MEP50 and its ability to methylate histone substrates, resulting in an altered gene expression profile in certain myeloproliferative neoplasms [186]. Conversely, cyclinD1/CDK4-mediated phosphorylation of MEP50 enhances methyltransferase activity of the PRMT5:MEP50 complex, increasing H4R3 and H3R8 methylation [2]. PTMs of substrates can also affect their ability to be modified by PRMT5. For example, histones H3 and H4, hypoacetylated in the context of SWI/SNF Sin3A/HDAC2, were dimethylated by PRMT5 much more efficiently in comparison with hyperacetylated histones [227]. There are also several instances in which different PRMTs act in opposition to each other and even inhibit one anothers activities. Histone H4K5 acetylation promotes H4R3 SDMA by PRMT5, while inhibiting ADMA deposition by PRMT1 [88]. An opposite and weaker association was identified with H3K16 acetylation, which decreases PRMT5 activity and enhances PRMT1 activity [88]. As mentioned previously, in another example, PRMT1 and PRMT5 competed for interaction

with E2F1, whereby the methylation marks that each deposited at slightly different arginine residues interfered with each others activities, affected E2F1 protein stability, and resulted in different cell viabilities [324]. These examples, among many others, highlight the dynamic interplay between different PTMs and how their combinatorial code can influence the scope of PRMT5 activities.

PRMT5 functions as part of numerous multiprotein complexes, and its interaction partners can also regulate and restrict its activity [160]. As a primary example, MEP50 is required for full PRMT5 methyltransferase activity. This complex then binds to other proteins which determine its substrate specificity, such as COPR5 [174] or BLIMP1, each of which can selectively methylate specific histones. PRMT5 contains three nuclear exclusion signals [263], but has been found to be present both in the nucleus and cytoplasm, depending on its interaction with specific binding partners and substrates. Furthermore, adaptor proteins RIOK1 and pICln, present in different cellular compartments, have been shown to bind to PRMT5 in a mutually exclusive fashion [119]. pICln (chloride channel nucleotide sensitive 1A) recruits Sm proteins to PRMT5, which are methylated and then incorporated into small nuclear ribonucleoproteins (snRNPs), while RIOK1 recruits nucleolin, which is known to be important for rRNA processing, for symmetric dimethylation. Taken together, these data offer a glimpse into how exquisitely PRMT5 activity is regulated in the cell by an intricate array of binding partners and modifications that affect the specificity and effects of PRMT5 methyltransferase activity.

Since lysine methylation is known to be reversed by demethylase enzymes of the KDM/LSD family, it stands to reason that arginine methylation is a dynamic post-translational modification that can be reversed. Until now, the only report of an arginine-specific demethylase identified the Jumonji-domain containing protein JMJD6 [45], but the function has been debated based on demethylase activity assays and structural analysis [28, 75]. Therefore, a bona fide arginine demethylase remains elusive.

■ 1.2.9 Readers of symmetrically dimethylated arginines

Methylarginine marks are signals that must be read by effector proteins or readers that can translate the signal into a functional outcome. The main readers of methylarginine marks are the Tudor domain containing family of proteins, some of which recognize methylarginines, while others bind methylysines [105]. SMN, encoded by the *SMN1*

(survival of motor neuron 1) gene, contains one Tudor domain. Loss of function point mutations in this domain have been found in patients suffering from Spinal Muscular Atrophy (SMA), an autosomal recessive neurodegenerative disease that results in motor neuron death [105]. SMN binds methylated spliceosomal core proteins and facilitates their assembly into snRNPs to enable splicing. SMN can bind both ADMA and SDMA marks, with a slight preference for the latter [187]. SPF30, another Tudor family member, is also involved in spliceosome maturation, and binds SDMA with a slightly higher affinity. Another prominent member of the Tudor family is SND1 (TSN-p100), which has been involved in transcriptional regulation, RNA processing, and alternative splicing. Zheng and colleagues demonstrated that when PRMT5 methylates E2F1, an interaction that is antagonized by PRMT1, SND1 binds the methylated arginines and then localizes to promoters of E2F target genes that promote survival and differentiation [324]. This localization of SND1, as determined by ChIP, is E2F1 dependent, as it was not present in E2F1 knockdown cells.

Aside from the Tudor family, a few other proteins have been shown to specifically bind symmetrically dimethylated substrates. As one example, WDR5 recognizes H3R2me2s on euchromatic promoters of genes expressed upon cell cycle exit and differentiation, and recruits MLL coactivator complexes, resulting in deposition of transcriptionally activating H3K4 trimethylation marks [204]. Meanwhile, asymmetric dimethylation of H3R2 by PRMT6 prevents binding of WDR5 and subsequent recruitment of chromatin modifying coactivator complexes. Together, these examples begin to uncover how the interpretation of the PRMT5 SDMA mark by different readers is connected to different functional outcomes.

■ 1.2.10 Roles for PRMT5 in splicing regulation

One of the earliest described roles of PRMT5 was as a component of the methylosome, a complex involved in spliceosomal assembly [95]. Since then, the known effects of PRMT5's involvement in splicing continue to expand, but a thorough mechanistic understanding of the steps in splicing that are impinged upon by PRMT5 activity and the effects of dysregulating this activity are still lacking.

Alternative splicing of precursor mRNAs encoded by a single gene offers eukaryotes the capacity to expand their transcriptome and proteome. Studies estimate that more than 90% of human genes are alternatively spliced [79]. Aberrant splicing has been

linked to diseases, including cancer, through direct and indirect mechanisms. These include alternative splicing of specific oncogenic isoforms, mutations in splice sites causing mis-splicing of tumor suppressor genes, mutations in core spliceosomal proteins and splicing factors, oncogenic stress, or mutations in epigenetic regulators.

Splicing of pre-mRNAs involves the recognition of the exon-intron boundaries via short sequence motifs, and subsequent removal of the non-coding intronic sequence by a series of transesterification reactions. More than 99% of the splicing in human cells is performed by the major spliceosome, a structure composed of U1, U2, U4, U5, and U6 small ribonucleoprotein complexes (snRNPs) and non-snRNP proteins [79, 167]. The U1snRNP binds the 5' splice site and U2snRNP recognizes the branch point, while a U2 auxiliary factor complex binds the 3' splice site. The U4/U6.U5 tri-snRNP is recruited to form the active spliceosome.

PRMT5 is known to methylate core spliceosomal proteins in the cytoplasm, modulating their ability to assemble into mature snRNPs. The snRNP core particles are composed of small nuclear RNA (snRNA) and seven Sm proteins (B, D1, D2, D3, E, F, G), as well as other proteins that are specific to each snRNP (reviewed in [91]). Assembly of the snRNP complexes begins in the cytoplasm, with translation of the Sm proteins. They do not exist as monomers, instead forming heterodimers and heterotrimers: SmD1/D2, SmB/D3, and SmF/E/G. The methylosome, composed of PRMT5:MEP50 and the chaperone protein pICln (encoded by CLNS1A), targets SmB, SmD1, and SmD3 for symmetric dimethylation [31, 95, 96, 202]. The Sm proteins form two separate higher order complexes, and the methylosome complex separately binds SmD1/D2/F/E/G (collectively called Sm5) and SmB/D3. Specifically, pICln appears to act as a chaperone for higher order Sm structure and proper snRNP assembly, preventing the premature association of Sm proteins with snRNA [47]. The SDMA post-translational modification increases the affinity of the Sm proteins for the Tudor domain containing SMN complex, dissociating them from pICln. The SMN complex loads the Sm proteins onto the conserved Sm site of the appropriate snRNA in a step-wise fashion, such that Sm5 binds first, followed by SmB/D3 to close out the ring. Following a few more RNA processing steps, the core snRNPs are then transported into the nucleus to mediate splicing as part of the spliceosome (reviewed in [91]). The SMN complex facilitates import and then dissociates from the snRNPs.

Several pieces of evidence suggest that PRMT5 could play roles in splicing that are separate from its methylation of Sm proteins. The first is that PRMT5, like other

PRMT family members, methylates numerous splicing factors other than the core Sm proteins [26, 51, 222]. Second, in *Drosophila*, loss of *dart5*, the homolog of *PRMT5*, did not affect snRNP assembly [112]. Lastly, a few reports have described that loss of PRMT5 across numerous species results in increased alternative splicing of elements with weak 5' splice sites [23, 71, 166, 243]. Therefore, it seems highly likely that PRMT5 could control other steps in the splicing pathway, either co-transcriptionally or post-transcriptionally. These roles remain to be explored and offer exciting new avenues of research.

■ 1.2.11 PRMT5 in development and determination of cell fate

Increasing evidence points to important roles for PRMT5 in maintenance of embryonic and adult stem cells [114]. PRMT5 was shown to be required for embryonic development, with a role in maintaining pluripotency in ES cells, while also repressing differentiation factors [276]. In a conditional knockout model, loss of PRMT5 in hematopoetic stem and progenitor cells led to an initial expansion, eventually followed by exhaustion and bone marrow aplasia [185]. In the brain, it appears that while PRMT5 plays a role in maintaining neural stem cells [53], it promotes oligodendrocyte differentiation [142]. A conditional knockout of PRMT5 in the central nervous system led to loss of neuronal progenitor cells, which could be partially rescued by p53 deletion [23]. A couple of complementary recent studies have found that PRMT5 is important for maintaining genome integrity during germline specification [163, 185]. During the global epigenetic reprogramming that occurs as part of germline development, PRMT5 maintains genome integrity by silencing retrotransposon elements, through H2A/H4R3me2s [163]. Later during germ cell development, PRMT5 maintains splicing fidelity and regulates survival [182]. The nuclear and cytoplasmic shuttling of PRMT5 was found to be concurrent with these functions. These results point to important roles for PRMT5 in stem cell maintenance.

■ 1.2.12 Diverse roles of PRMT5 in cancer

PRMT5 is dysregulated across a wide variety of cancers (Figure 1.3), and often correlated with poor prognosis in many solid and blood-borne tumors [263]. The majority of cases involve amplifications, with a much smaller proportion displaying deletions. Mutations in PRMT5 have also been documented in human tumors, although the func-

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tional consequences of these mutations have not been tested. PRMT5 has been linked to promoting neoplastic growth through several different mechanisms, such as controlling cellular proliferation, cell cycle, cell death, and invasiveness. As the link between PRMT5 and cancer continues to strengthen, emerging studies are investigating the possibilities for therapeutic inhibition of PRMT5.

One of the earliest analyses of the functional consequences of PRMT5 mediated transcriptional repression revealed that PRMT5 overexpression led to downregulation of tumor suppressor genes ST7 and NM23 [226]. Since then, the role of PRMT5 in numerous processes important for tumorigenesis, such as the DNA damage response, cell cycle regulation, epithelial to mesenchymal transition, cellular signaling cascades, and inflammation have been described. Although PRMT5 has been investigated in the context of numerous cancer types, it has proven difficult to dissect the exact pathways leading to the development or maintenance of tumors for a protein with such a dense network of interaction partners, and it has become increasingly clear that its effects are highly cell type and context specific.

Our understanding of the oncogenic functions of PRMT5 in hematologic malignancies has greatly expanded over the past couple of years. Li and colleagues reported that PRMT5 is required for lymphomagenesis, downstream of several oncogenic drivers, namely cyclin D1, c-MYC, NOTCH1, and MLLAF9 [182]. PRMT5 was also overexpressed in primary lymphoma samples [55], and found to upregulate its own expression in lymphoma cells through a PRMT5/p65/HDAC3 repressive complex that restricted expression of a PRMT5-targetting miRNA [4]. Furthermore, the novel PRMT5 inhibitor EPZ015666 was first tested on MCL xenograft models, resulting in reduced tumor burden [44]. In an elegant report linking lymphomagenesis and splicing, Koh and colleagues found that PRMT5 is important for Eμ-myc driven tumor development and maintenance, as myc overexpression drives dependence on high levels of PRMT5 to maintain splicing fidelity [166].

High levels of PRMT5 expression have also been reported in numerous solid tumors, often correlated with aggressive disease. In lung tumors, PRMT5 was found to be overexpressed patient samples from numerous subtypes [254, 255], with higher levels of PRMT5 and MEP50 being associated with decreased survival in non-small cell lung cancer [263]. PRMT5 knockdown successfully reduced tumor growth in a lung cancer xenograft model [117], although its effects in autochthonous models have not been reported. Additionally, high levels of PRMT5 have been noted in human melanomas,
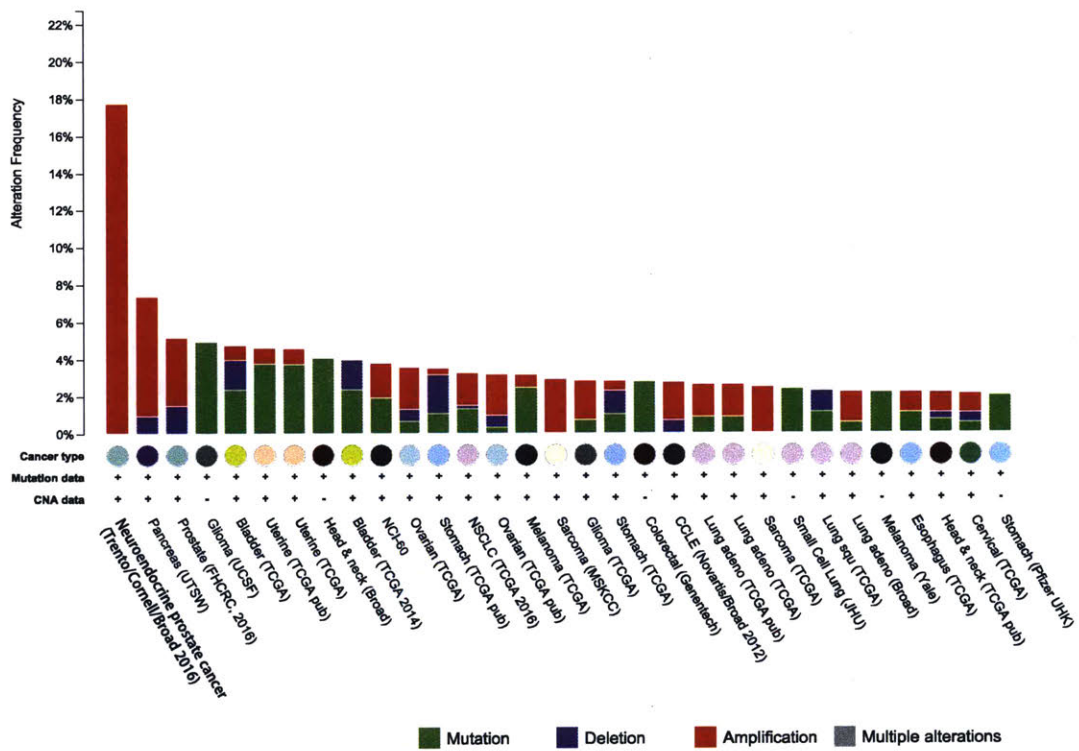
Figure 1.3. PRMT5 alterations in cancer. PRMT5 is frequently altered in a wide variety of tumor types. The distribution of copy number alterations (amplifications and deletions), as well as mutations, are displayed. These results are based on data generated by the TCGA Research Network: http://cancergenome.nih.gov/, obtained from cbioportal.org.

ovarian, and prostate tumors [14, 116, 217]. Although PRMT5 functions in both nuclear and cytoplasmic compartments, and staining in primary tumor samples is often heterogeneous, there is evidence to suggest that high cytoplasmic expression is often correlated with tumor grade [217, 255].

PRMT5 has also been shown to regulate another tumor suppressor, p53, through several different direct and indirect mechanisms. Under DNA damage conditions in U2OS osteosarcoma cells, PRMT5 was found to methylate p53 on arginine residues contained in its oligomerization domain, altering its promoter specificity and reducing apoptosis [152]. Scoumanne et al. described another mode of PRMT5 dependent p53 regulation, noting that PRMT5 regulates p53 translation, possibly through EIF4E [250]. Here, PRMT5 knockdown resulted in decreased EIF4E protein levels and decreased cellular proliferation, which was rescued by EIF4E overexpression. Therefore, PRMT5 broadly functions to suppress multiple tumor suppressor pathways, providing clues about its role as a potential oncogene.

The RB tumor suppressor pathway is one of the most highly deregulated pathways in human tumors. PRMT5 acts upon various components of this pathway, invariably driving increased capacity for cellular proliferation and tumorigenesis. In lymphoid cell lines, PRMT5 overexpression led to increased H4R3me2s and H3R8me2s at promoters of RB family members, resulting in their transcriptional repression. PRMT5 knockdown in these lines led to decreased cell proliferation [295]. As indicated earlier, the La Thangue group showed that E2F1 is methylated in a competitive manner by PRMT1 and PRMT5 and each of these modifications has different functional outcomes [54, 324]. PRMT5-mediated methylation of E2F1 leads to cell cycle progression, while PRMT1-mediated methylation under DNA damage conditions results in E2F1 dependent apoptosis. In a different study, Aggarwal et al. demonstrated that cyclin D1 regulates PRMT5 activity, via CDK4-mediated phosphorylation of MEP50 [2]. This modification increased PRMT5 activity, resulting in increased H4R3me2s and subsequent repression of CUL4A/B expression. These components of the E3 ubiquitin ligase complexes assist in the degradation of DNA replication licensing factor CDT1. The authors had previously shown that overexpression of CDT1 during S phase allows for DNA re-replication, leading to genomic instability and subsequent increased mutational burden [1]. Taken together, these results illustrate how PRMT5 can impinge on the RB pathway from multiple angles, and promote tumor cell proliferation.

One of the hallmarks of cancer is the epithelial to mesenchymal transition (EMT), a

process that enables cells to lose polarity and cell-cell adhesions, while gaining migratory and invasive phenotypes that favor tumor cell dissemination and metastasis [124]. This change involves loss of E-cadherin (*CDH1*) expression. PRMT5 has been shown to be recruited to the EMT transcription factor SNAIL, together with the repressive complex AJUBA, resulting in repression of the *CDH1* gene in HEK293 cells [137]. Additionally, overexpression of PRMT5 in NIH-3T3 cells induced anchorage-independent growth [226]. In a recent report characterizing the involvement of TGF-β driven EMT, Chen et al. provide evidence for a complex mechanism of PRMT5-orchestrated transcriptional regulation of TGF-β responsive genes important for mediating EMT [49]. The authors demonstrate that in response to TGF-β treatment in lung cancer cells, PRMT5/MEP50 simultaneously targets H4 and H3, with different outcomes. H4R3 symmetric dimethylation results in subsequent transcriptional repression of TGF-β silenced EMT genes, while H3R2 monomethylation and symmetric dimethylation results in transcriptional activation of TGF-β activated EMT genes, via the recruitment of WDR5. Although the association of H3R2me2s with this component of the MLL complex has been previously reported [204], this is the first report of H3R2me1 function. Overall, these studies define a role for PRMT5 in TGF-β driven EMT.

The post-translational modifications deposited by PRMT5 also play roles in signal transduction cascades, pathways whose components are often found to be mutated in cancers. For example, PRMT5 can regulate receptor tyrosine kinase signaling, restricting ERK activity. PRMT5-mediated methylation of the epidermal growth factor receptor (EGFR) at R1175 stimulates EGF-mediated trans-auto-phosphorylation at Y1173, which leads to binding of phosphatase SHP1 (PTPN6) and subsequent attenuation of downstream signaling [138]. Inhibition of this modification resulted in increased proliferation, migration and invasion of an epidermoid carcinoma cell line. PRMT5 also methylates both BRAF and CRAF in response to certain growth factors in PC12 cells, decreasing their stability, activity and subsequent ERK activation [9].

A couple of recent promising studies have outlined that PRMT5 inhibition might be particularly beneficial in tumors lacking the metabolic enzyme methylthioadenosine phosphorylase (MTAP) [172, 198]. As part of the methionine and adenine salvage pathways, MTAP is involved in the generation of the PRMT5 cofactor SAM. MTAP is frequently deleted in cancers, for example in 53% of glioblastomas (GBMs) and 26% pancreatic tumors, due to its proximity on chromosome 9 to the tumor suppressor gene *CDKN2A*. Using pooled shRNA screens against hundreds of cell lines, both studies

identified that the viability of MTAP negative cell lines was impaired by knockdown of PRMT5 and several of its cofactors, such as MEP50. MTAP deficient cells built up the metabolite methylthioadenosine (MTA), leading to a partial inhibition of PRMT5 methyltransferase activity. Cells were further sensitized to PRMT5 loss in this hypomorphic state [172, 198]. These results show that MTAP deleted tumors may be selectively vulnerable to PRMT5 inhibition, although they do caution that MTAP negative cell lines show a range in the extent of growth reduction in response to PRMT5 loss and therefore MTAP status may not be sufficient to determine sensitivity to PRMT5 inhibition.

Importantly, PRMT5 expression levels have been found to be higher in numerous tumor types compared to surrounding normal tissues and is often an indicator of advanced disease [14, 117, 217, 254, 255, 312], speaking to the requirement for PRMT5 activity in tumorigenesis, and indicating its potential utility as a biomarker. Furthermore, all of these studies have increased the interest in small molecule inhibitors for targeting PRMT5. Recently, an inhibitor with high specificity, oral bioavailability, and nanomolar IC50 has been reported [44], opening the door for studies to assess the relevance of PRMT5 as a therapeutic target in a variety of cancers.

■ 1.3 Genetic and Epigenetic Dysregulation in Glioblastoma

Diffuse gliomas represent the most common primary brain tumors in adults [146]. These neoplasms display diffuse infiltration of tumor cells into the neuropil, and are categorized as low grade glioma (LGG) or glioblastoma (GBM). LGGs are classified according to their histopathological characteristics as astrocytomas, oligodendrogliomas, or of mixed origin, oligoastrocytomas [146, 190]. These tumors are further stratified by grade, depending on their mitotic index and nuclear atypia, as being WHO Grade II and III. GBMs, the most frequent of gliomas, are grade IV tumors that either arise de novo as primary tumors, or by progressing to a fully malignant state from LGG, known as secondary GBM. The incidence of GBM is 3.2 per 100,000 people in the US, and it increases with age, with a median age of diagnosis of 64 [223]. These tumors carry a dismal prognosis, with a median survival of 14.6 months and 5-year survival of less than 10 percent, with standard treatment methods [265], highlighting the need for novel therapeutic approaches.

■ 1.3.1 Conventional therapeutic options for glioblastoma

GBM patients currently undergo surgical resection whenever possible, and are subsequently treated with a combination of radiation therapy and the chemotherapeutic temozolomide (TMZ). Prior to the early 2000s, glioblastoma patients were treated with radiation therapy and nitrosoureas, DNA alkylating agents such as carmustine (BCNU) or lomustine (CCNU) [110]. However, numerous clinical trials did not show any consistent benefit of using adjuvant chemotherapy compared to radiation therapy alone [265]. A retrospective review of 12 randomized trials showed a small but significant survival benefit [110], but the use of these chemotherapies was often associated with severe side effects.

The DNA methylating agent TMZ was developed in the 1980s and found to have favorable bioavailability, distribution to the brain, and a tolerable side effect profile [262]. In 2005, Stupp and colleagues reported the result of a European and Canadian phase III clinical trial for GBM patients, comparing radiotherapy alone against radiotherapy and concomitant and adjuvant TMZ [265]. These results demonstrated a significant improvement in median survival, from 12.1 to 14.6 months, and an increased two-year survival from 10.4% to 26.5% in the cotreated group [265]. This paved the way for large-scale adoption of TMZ for the treatment on GBM, and an updated analysis of

the trial a few years later demonstrated a significant increase in the five-year survival rate from 1.9% to 9.8% [264]. TMZ is now used as the standard chemotherapy for GBM [145].

TMZ acts by methylation of DNA bases and activates several cellular DNA repair pathways. The major molecular targets of TMZ-induced methylation are the N7 position of guanine (N7-G, 70%), N3 of adenine (N3-A, 9%), and O6 of guanine (O6-G, 6%) [321]. While methylated N7-G and N3-A are substrates for the base excision repair (BER) pathway, the most toxic TMZ adduct, methylated O6-G (O6-meG), is normally repaired by the suicide enzyme O6-methylguanine-DNA-methyltransferase (MGMT, Figure 1.4). If not resolved, O6-meG leads to a thymine mismatch and a point mutation during replication. The mismatch repair (MMR) pathway recognizes this lesion and attempts to correct it. However, this mechanism only acts on the newly synthesized daughter strand, without repairing the original O6-meG on the template strand. This leads to repetitive and unsuccessful attempts to fix the lesion, resulting in persistent gaps in the DNA and double stand breaks upon further replication [97]. Such clasto-genic lesions will lead to apoptosis if downstream repair pathways fail. Therefore, a simple alkylating lesion can become toxic to rapidly proliferating tumor cells [157].

Due to the prominent role of MGMT in efficacy of TMZ, MGMT expression was proposed as a biomarker for treatment response [132]. Epigenetic silencing of MGMT by promoter methylation is found in approximately half of GBM patients [132, 133], and several studies reported that TMZ-treated patients harboring MGMT promoter methylation showed a significant improvement in median survival (21.7 months versus 15.3 months) [133]. As a result, MGMT promoter methylation status is now used for patient stratification in clinical trials [279]. However, there is no consensus on the best method to collect tissue and determine MGMT methylation patterns in patients [278, 279]. Other studies have also shown that there is not a clear correlation between MGMT promoter methylation status and mRNA or protein expression [171, 175]. Therefore, the predictive value of MGMT promoter methylation for treatment outcome remains unclear. New molecular subclassification of GBM tumors has recently determined that while MGMT promoter methylation is found in about half of patients, it is predictive of survival in only one subtype, known as the classical subgroup [35]. Altogether, although the current use of unselective conventional therapies has resulted in modest improvement in the short term survival of GBM patients, new approaches are warranted for developing novel therapeutic strategies with the aim of improving long term survival.
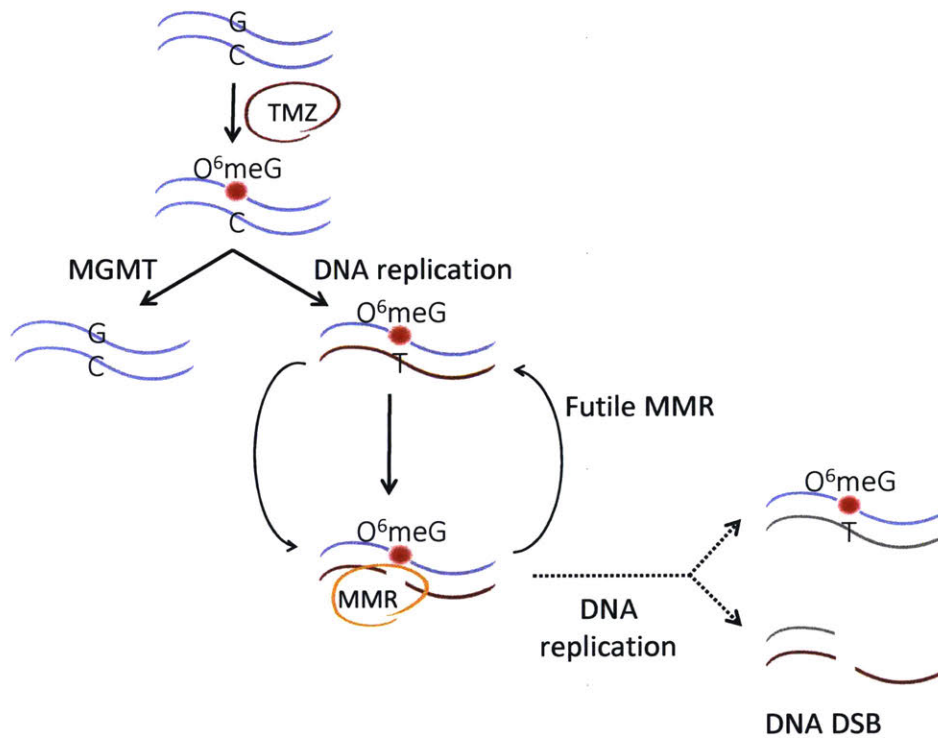
Figure 1.4. Mechanism of action of temozolomide (TMZ). Of the three major TMZ-induced adducts, the most toxic is methylated O6-G (O6meG). This lesion is normally repaired by the suicide enzyme O6-methylguanine-DNA-methyltransferase (MGMT). If not resolved, O6meG leads to a thymine mismatch and a point mutation during replication. The mismatch repair (MMR) pathway surveys the daughter stand and recognizes the mismatch. In a process known as "futile MMR", it repeatedly and unsuccessfully attempts to fix the lesion. Upon further rounds of DNA replication, this mismatch can persist or lead to DNA double strand breaks (DSB).

■ 1.3.2 Molecular subclassification uncovers interplay between genetics and epigenetics

Gliomas represent a heterogeneous class of tumors, and their distinction based on histopathological characteristics and grade conceals their true molecular diversity. A more thorough understanding of the key genetic and epigenetic alterations would allow for more distinct stratification of patients and clearer insight into which patients would benefit from different therapies. In the past decade, the advent of high throughput genomic analysis technologies and careful cataloging of hundreds of tumor samples by consortia such as TCGA have allowed for large scale profiling analyses. These studies have led to molecular subclassification of gliomas based on genomic, epigenomic, and transcriptional signatures [144].

One of the first efforts to define molecular signatures was undertaken by Phillips and colleagues [232], who classified grade III and IV malignant tumors into three subclasses based on their gene expression patterns: proneural, proliferative, and mesenchymal. The proneural group displayed neuronal lineage markers and was associated with a favorable prognosis, while the other two subclasses expressed signatures of proliferation, mesenchymal tissues and angiogenensis, and were associated with poor prognosis. Our knowledge of the molecular basis of GBM rapidly expanded with publication of the first TCGA glioblastoma analysis, detailing the most commonly mutated genes and pathways in a sample of 206 tumors. Dysregulation of the RB pathway, p53 pathway, and receptor tyrosine kinase signaling was found in almost all tumors, with 74% containing aberrations in all three. The most common alterations involved mutations or homozygous deletions in CDKN2A/B, PTEN, and TP53, and mutations or amplifications in EGFR. Molecular classification of the TCGA data subsequently revealed 4 tumor classes linked to distinct neural lineages and therapeutic response [290]. The classical, mesenchymal, proneural, and neural types expressed gene signatures similar to astrocytes, microglial and mesenchymal cells, oligodendrocytes, and neurons, respectively. Furthermore, three subclasses were strongly associated with genetic abnormalities in different genes: classical with EGFR, mesenchymal with NF1, and proneural with PDGFRA and IDH1/2. This study also demonstrated the predictive power that molecular subclassification could have even for conventional therapies, with the classical and mesenchymal subtypes showing improved survival with more intensive therapy, while the proneural class fared best, regardless of treatment intensity.

Armed with a better understanding of the genetic events driving different subclasses of GBM, subsequent investigations examined stratification based on epigenetic modifications, starting with DNA methylation. To this end, Noushmehr et al. looked at genome wide promoter DNA methylation of a growing TCGA dataset [218]. Reminiscent of a CpG island methylator phenotype (CIMP) subgroup first described in colorectal cancer [281], this study revealed the existence of a glioma CpG island methylator phenotype (G-CIMP). This pattern is correlated with the proneural GBM subgroup, enriched in secondary and recurrent GBMs, and associated with younger patient age. Notably, these tumors contain mutations in the metabolic enzymes isocytrate dehydrogenase 1 or 2 (IDH1, IDH2) and are associated with improved outcome compared to C-GIMP negative tumors [281]. The G-CIMP phenotypes and IDH mutations also frequently occur in lower-grade gliomas. These findings, together with several others [65, 89, 286, 300, 310], revealed an intricate link between genetic alterations and epigenetic changes in GBM. Mutations in IDH1 and IDH2 result in the production of a neomorphic enzyme that produces an oncometabolite, 2-hydroxygluterate (2-HG). This new product inhibits the demethylase activity of TET2, leading to an increase in DNA methylation, ultimately resulting in G-CIMP. These mutations appear to upregulate neuronal stem cell markers in astrocytes, indicating that the resulting epigenetic remodeling can affect cellular differentiation [286]. Hypermethylation can have striking effects on chromosomal topology, disrupting domain boundaries that are normally separated by insulator elements [92]. An elegant report recently demonstrated that hypermethylation reduces CTCF insulator protein binding to DNA, resulting in a breakdown of boundary structure and leading to interactions of enhancers with oncogene promoters. Specifically, this led to activation of PDGFRA, whose aberrant activation increased proliferation in glioma neurosphere culture. Treatment of IDH1 mutant cells with demethylating agent 5-azacytidine restored CTCF function and downregulated PDGFRA expression [92]. Interestingly, 2-HG can also inhibit specific histone demethylases [310] and block differentiation [89, 191]. Therefore, interrogation of the DNA methylation patterns in GBM has revealed a novel oncogenic driver that crosses the boundaries between metabolic and epigenetic regulation and has important implications for the subclassification of gliomas.

Over the past few years, several studies have offered insight into how DNA methylation patterns can help to stratify not only adult GBM, but also LGG and pediatric GBM. Brennan and colleagues analyzed a set of 543 GBM patients as part of TCGA
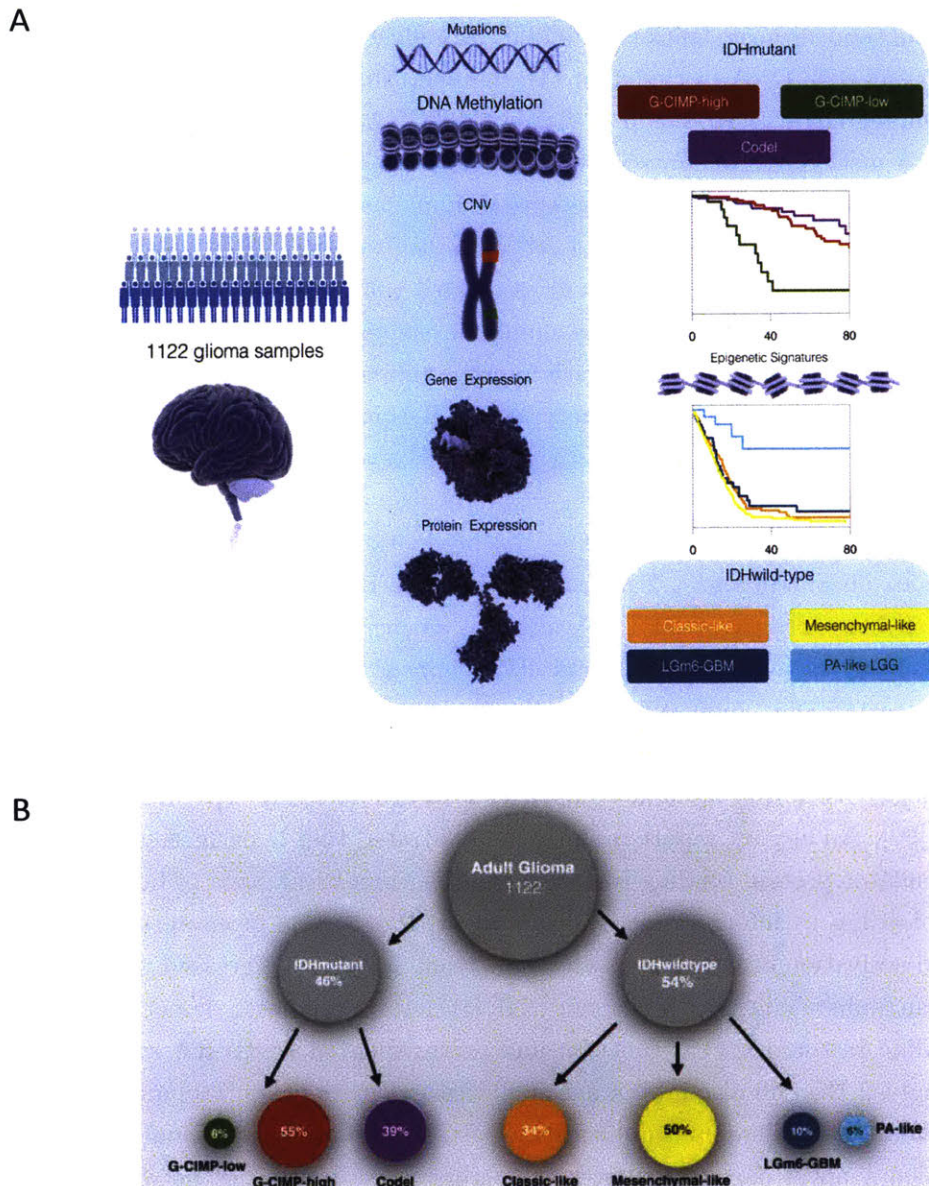
Figure 1.5. New classification scheme for gliomas. (A) Tumors from a large number of human glioma patients were profiled for several molecular characteristics such as mutations, DNA methylation, copy number variants, gene and protein expression. This analysis led to a novel subclassification scheme for gliomas based on DNA methylation patters, which stratified patients broadly into IDH mutant or wild type tumors, and further into three or four subclasses, respectively. These groups are associated with different survival outcomes. (B) Scheme showing the percentage of patients that stratify into each of the different categories. (Modified from [41])

and noted that DNA methylation stratified patients into 6 subclasses. This included an IDH mutant, G-CIMP+ group that was associated with the most favorable survival outcome. Other studies demonstrated that most LGG tumors were mutant for IDH and those with WT IDH1 showed a high level of similarity to GBM both molecularly and clinically [39, 81]. The most recent analyses replaced grade distinctions between GBM and LGG with classification by DNA methylation. This allowed the authors to recognize two broad classes based on IDH status [41, 81]. Within the IDH mutant group, further subclassification outlined G-CIMP high, G-CIMP low, and tumors containing a 1p/19q co-deletion, with G-CIMP low showing the lowest median survival (Figure 1.5). Meanwhile, the IDH WT tumors were further subdivided into 4 groups with different expression patterns and clinical outcomes. In a cohort of pediatric and adult GBM patients, mutations in H3F3A, encoding histone variant H3.3, were associated with different methylation patterns and a younger patient population [267], echoing findings of histone mutations in pediatric GBM [248]. Mutually exclusive with mutations in IDH, mutations in H3F3A co-occur with genetic hits in ATRX and DAXX, known to complex together and contribute to an alternative lengthening of telomere (ALT) phenotype [248, 267]. Interestingly, the majority of adult GBM tumors contain alterations in the TERT promoter or ATRX, indicating that telomere maintenance is important in GBM. Despite these observations, the significance of telomere length to gliomagenesis and effect on survival remains to be clarified [41, 101, 162, 266].

In summary, these recent efforts demonstrate that integrated genomic and epigenomic analyses involving DNA methylation, gene mutations, copy number alterations, as well as gene and protein expression can drive refinement of glioma classification across age and tumor grade. Such grouping would be beneficial for guiding more targeted clinical trial design, as well as discovery of biomarkers and development of specific treatment strategies. The identification of several molecular subgroups argues that blanket treatment with a non-selective agent such as radiation and temozolomide may be inefficient in such a heterogeneous tumor type [266].

■ 1.3.3 Emerging therapeutic strategies in GBM

Although our understanding of the molecular mechanisms underlying gliomagenesis has greatly improved, targeted therapies have not yet found their way into large scale clinical practice.

Several reasons have been discussed for the current lack of success of targeted and epigenetic therapies [50, 266]. First, systemic treatments of GBM tumors face the unique challenge of penetrating the blood brain barrier, which regulates the passage of signaling molecules, toxins, and consequently drugs, into the brain parenchyma [43]. Second, the lack of substratification of patients in clinical trials can lead to any potential effects being masked when diluted over a heterogeneous patient population. Third, tumors are notoriously efficient at eventually developing therapeutic resistance, whether due to the outgrowth of pre-existing resistant clones or the emergence of novel drug resistant cells [80].

The only targeted agent currently approved by the FDA is the anti-angiogenic therapy bevacizumab [50]. In preclinical models, this monoclonal antibody targeting vascular endothelial growth factor (VEGF-A) has been shown to decrease angiogenesis, one of the hallmark features of GBM, resulting in temporary restoration of tumor vasculature [151]. Despite its initial success in increasing progression-free survival in phase II trials for patients with recurrent GBM [93, 170], two recent phase III studies did not find that bevacizumab had any effect on overall survival as first line therapy in combination with radiation and TMZ for patients with newly diagnosed GBM [52, 107].

One potential therapeutic strategy that has been studied extensively is targeting EGFR, one of the most frequent alterations in GBM, found in 57% of patients [133]. Small molecule EGFR inhibitors that have worked well in other solid tumor types have not been successful in GBM [224]. Cetuximab, an antibody targeting wild type EGFR, did not demonstrate an effect on survival in a phase II trial that stratified recurrent high grade glioma patients by EGFR amplification status [216]. Other areas of active investigation include antibodies or vaccines against EGFRvIII, a mutant version of the receptor [50, 224].

Epigenetic therapies are currently being explored in clinical trials involving glioblastoma patients. A retrospective study of patients taking the anti-epileptic drug valproic acid, an HDAC inhibitor, during the course of their radiation therapy found that it was associated with improved survival [15]. The recently published results of a phase II trial of valproic acid with concomitant TMZ and radiation showed that the therapy was well tolerated in GBM patients and that outcomes may show improvement, warranting a phase III trial [169]. The DNA demethylating agent 5-azacytidine, approved for use in myelodysplatic disorders [37], has shown efficacy in a preclinical model of IDH mutant glioma [27] and is currently being tested in GBM and many other solid tumors in a

phase I trial [42]. Given the new appreciation for the high frequency of IDH mutation in glioma and great strides in understanding its mechanism of tumorigenesis, recent efforts have been aimed at targeting mutant IDH enzymes, which are found not only in glioma but also AML and a few other malignancies. Small molecule inhibitors and vaccines are currently being explored in Phase I clinical trials in glioma and IDH mutant positive malignancies [213, 219]. Furthermore, a phase IIa clinical trial in GBM patients using a small molecule inhibitor of the BET family of chromatin readers, OTX015, has recently been completed, with results pending [221].

Several lines of cancer immunotherapies are being tested in gliomas at this time. Aside from the aforementioned vaccines targeting specific proteins mutated in gliomas, several dendritic cell vaccines are currently underway [50]. Excitement over immunotherapies has increased with the recent approval of two checkpoint blockade inhibitors as first line therapy for metastatic melanoma (reviewed in [275]). These antibodies against PD-1 and CTLA-4 are currently being tested in phase III clinical trials in recurrent or newly-diagnosed GBM, head to head with TMZ. Lastly, adoptive cell therapies are also being tested using anti-EGFRvIII chimeric antigen receptor (CAR) T cells. The aggressive nature of GBM and the heterogeneity of tumors assimilated under this one umbrella term has resulted in few novel therapeutic strategies targeting these tumors in the past couple of decades. It is likely that effective treatment will require multiple modes of therapy and the exact approach will be informed by molecular characteristics of the tumor, as well as clinical phenotypes. Our understanding of the actions of epigenetic regulators in cancer is expanding rapidly, offering us a better understanding of the potential therapeutic benefits of epigenetic therapies. Ultimately, these therapies may prove to be useful for remodeling the epigenetic landscape of tumor cells, and sensitizing tumors to either targeted therapies or conventional chemotherapies [80, 156, 319].

■ 1.3.4 Known roles for PRMT5 in GBM

PRMT5 has been studied in the context of GBM in only a handful of previous reports. Protein expression of PRMT5 was found to increase with tumor grade, while being minimal in normal brain [123, 312]. Furthermore, levels of PRMT5 protein expression were negatively correlated with patient survival. PRMT5 knockdown in different human GBM lines had anti-proliferative effects and resulted in apoptosis in a p53 independent manner. One of the genes that was upregulated upon PRMT5 loss was tumor suppressor ST7, and PRMT5 was shown to be recruited to the ST7 pro-

moter. Furthermore, PRMT5 knockdown in an orthotopic xenograft model resulted in increased survival [312]. Recently, experiments in serum-deprived GBM neurospheres versus adherent and more differentiated cultures demonstrated that PRMT5 regulates self-renewal via the PTEN-Akt pathway [13]. PRMT5 loss led to PTEN-dependent senescence in the neurospheres and was required for survival in adherent cells. In xenograft models derived from both cell types, PRMT5 knockdown improved survival. Together, these studies point to a role for PRMT5 in GBM and advocate for further investigation into the mechanisms of PRMT5 function and its potential as a therapeutic target in GBM.

■ 1.4 Conclusion

Our understanding of epigenetics has greatly expanded over the past 30 years and our appreciation for the diverse activities of epigenetic regulators and their contributions to cancer is rapidly expanding. The PRMT family of arginine methyltransferases, and specifically, PRMT5, is emerging as an important contributor to tumorigenesis across a variety of tumor types. At a time when the epigenetic contributions to GBM pathology are being increasingly explored, PRMT5 comes into focus as an ideal candidate for further exploration and therapeutic targeting.

Chapter 2

In vivo RNAi screen uncovers PRMT5 as a critical mediator of tumor growth in glioblastoma

Monica Stanciu, Christian Braun, Paul Boutz, Rachit Neupane, Michael Hemann, Jacqueline Lees

M.S. and C.B. conducted *in vitro* and *in vivo* experiments. M.S. performed gene expression pathway analyses. C.B. performed cell line screen. P.B. analyzed gene expression data, including all splicing analyses. R.N. contributed to Figure 2.8C. M.S., C.B., M.H., J.L. designed the study and analyzed the data. M.S. and J.L. wrote the paper.

■ 2.1 Abstract

GIVEN the growing appreciation for the role of epigenetics in glioblastoma (GBM), we performed an *in vivo* shRNA screen to identify epigenetic modulators of tumor growth. We identified PRMT5, a type II protein arginine methyltransferase, whose oncogenic roles in several tumor types are emerging, as being differentially required for tumor growth. We demonstrate that both genetic depletion and pharmacological inhibition with a new first in class PRMT5 inhibitor results in loss of cell viability and leads to lifespan extension in an *in vivo* GBM model. We describe a putative function for PRMT5 in regulation of GBM cell fate and define a critical role for PRMT5 in modulating splicing of a recently recognized class of intronic sequences that uniquely regulate gene expression. Furthermore, we demonstrate that despite the pleiotropic roles of PRMT5, resistance to PRMT5 inhibition is most strongly determined by the expression level of genes involved in RNA splicing across a range of tumor cell types. Together, these results highlight the importance of PRMT5 in GBM and demonstrate that using a small molecule inhibitor targeting its methyltransferase activity can lead to a significant effect on survival. Furthermore, our results suggest a requirement for PRMT5 regulated splicing across different tumor cell types, possibly owing to its ability to rapidly modulate gene expression through this function.

■ 2.2 Introduction

Glioblastoma (GBM) is a largely intractable disease whose molecular foundations are the subject of intense study, with the goal of developing more targeted therapeutic regimens leading to improved survival. Currently, GBM treatment consists of radiation therapy with concomitant or adjuvant chemotherapy with temozolomide (TMZ). The contributions of global epigenetic remodeling of the chromatin landscape to GBM initiation and progression are being increasingly explored. The advent of large scale studies such as The Cancer Genome Atlas (TCGA) have greatly accelerated our appreciation of the breadth of epigenomic alterations in GBM [35, 41]. For example, DNA methylation patterns were shown to form the basis for molecular subclassification across adult diffuse gliomas of all grades [41], and these different subclasses are linked with specific molecular characteristics and survival outcomes. Furthermore, 46% of GBMs contained at least one non-synonymous mutation in a chromatin modifying gene [35]. These studies, along with many others [92, 99] highlight the undisputable importance of studying the role of the epigenome for a more complete understanding of GBM biology and the development of potential treatment strategies.

Deregulation of factors involved in RNA splicing is increasingly being recognized across a variety of tumor types (reviewed in [79]). These proteins can act as tumor suppressors or proto-oncogenes, affecting both constitutive and alternative splicing [69, 98, 126, 196, 240]. Interestingly, novel roles for epigenetic modulators in splicing regulation have recently been described. For example, MRG15 is a chromodomain containing protein that binds H3K36me3 marks and modulates alternative splicing by recruiting the splicing factor PTB [193]. Loss of SETD2, a histone methyltransferase, in renal cancer results in increased chromatin accessibility and splicing alterations in a quarter of expressed genes [256]. These results illustrate the importance of splicing in cancer and the blurring of boundaries between epigenetic and splicing roles.

Protein arginine methyltransferase 5 (PRMT5) is an epigenetic regulator that is indispensable for mammalian development [276] and whose role in cancer has become the focus of intense investigation in the last few years [263]. PRMT5 is overexpressed in a variety of solid and hematologic malignancies including lung and ovarian tumors, melanoma, and lymphoma [14, 55, 217, 254, 255, 303]. A member of the diverse family of protein arginine methyltransferases, PRMT5 is the major enzyme responsible for symmetric dimethylation of arginine residues (SDMA) on both histone and non-histone

proteins and its post-translational modifications target proteins across a wide range of cellular functions (reviewed in [20]). The effects of PRMT5 on gene expression are mediated via its epigenetic role as a histone modifier and transcriptional co-activator and co-repressor, as well as through its involvement in mRNA splicing.

The known roles for PRMT5 in splicing point to its involvement in the methylosome, a complex required for the assembly of small ribonucleoproteins (snRNPs) in the cytoplasm. Together with its binding partner pICln (CLNS1A), PRMT5 recruits Sm proteins, and specifically methylates SmB, SmD1, and SmD3. This modification is then thought to facilitate the transfer of Sm proteins to the Tudor domain containing SMN complex, which guides their assembly onto snRNAs and the formation of a complete snRNP complex [91]. Furthermore, deep sequencing revealed that PRMT5 loss leads to modifications in constitutive and alternative splicing events [23, 71, 166, 243].

PRMT5 was recently found to be overexpressed in GBM patient samples [123, 312], and shRNA mediated knockdown was shown to extend survival in GBM xenograft models [13, 312]. With mounting evidence for its critical role across a variety of tumor types, PRMT5 has emerged as a highly sought-after therapeutic target. A novel selective small molecule inhibitor for PRMT5 enhanced lifespan in xenograft models of mantle cell lymphoma [44]. RNA interference (RNAi) loss of function screens have been successfully used as an unbiased method of identifying novel regulators of tumorigenesis and therapy response [22, 36, 201, 235, 320, 325]. Although technically challenging, *in vivo* shRNA screens offer the unique opportunity to study gene function in a physiologically relevant context, as opposed to cell-based screening assays [201].

In the present study, we report the results of parallel *in vivo* and *in vitro* shRNA screens to identify epigenetic mediators affecting tumor growth and response to TMZ. Our results provide evidence for PRMT5 involvement in this disease and demonstrate *in vivo* survival benefits of inhibiting PRMT5 using a small molecule inhibitor. We describe a dependence on splicing regulators in response to PRMT5 inhibition that extends across a broad range of tumor cell types and identify a novel role for PRMT5 in regulating a specific class of introns.

■ 2.3 Results

■ 2.3.1 GL261 orthotopic transplantation tumors resemble human glioblastoma tumors

To perform a robust loss of function *in vivo* screen with a large pooled shRNA library in an orthotopic setting, we first set out to choose a system consisting of GBM cells that could be easily grown *in vitro* and transplanted intracranially with great reliability. We selected the murine GL261 GBM cell line, which was derived from a glioma generated by the implantation of carcinogenic methylcholanthrene pellets into the brain of a C57BL/6 mouse [215]. By orthotopically injecting these cells into syngeneic C57BL/6 mice, tumors were generated in immunocompetent hosts in the context of a physiologically relevant tumor microenvironment. We first confirmed that this model system recapitulates important histopathologic and mutational features of human tumors. Intracranial transplantation of GL261 cells into syngeneic hosts resulted in highly aggressive and infiltrative tumors, which, like their human counterparts, formed invasive projections into the neuropil (Figure 2.1A). These tumors were highly vascularized and displayed astrocytic features, as previously described [215]. Tumor development was rapid and reliable, with complete penetrance and a median survival of 23 days post implantation (Figure 2.1B). Treatment of the mice with TMZ significantly extended lifespan, although the animals invariably succumbed to the disease. We found that these cells contained a mutation in the DNA binding domain of p53 and an activating mutation in Kras, confirming what had been previously reported [271]. In human glioblastoma, aberrations in the p53 tumor suppressor pathway are found in 86% of patients, while TP53 itself is mutated in 28% of tumors [35]. While only 1% contain mutations in RAS, receptor tyrosine kinase signaling is altered in 90% of GBM, with mutations in EGFR, PDGFR and NF1 all converging on this signaling pathway [35]. These results demonstrate that the GL261 model system combines a syngeneic tumor model with a mutation spectrum that is relevant to human disease, leading to tumors that resemble their human GBM counterparts.
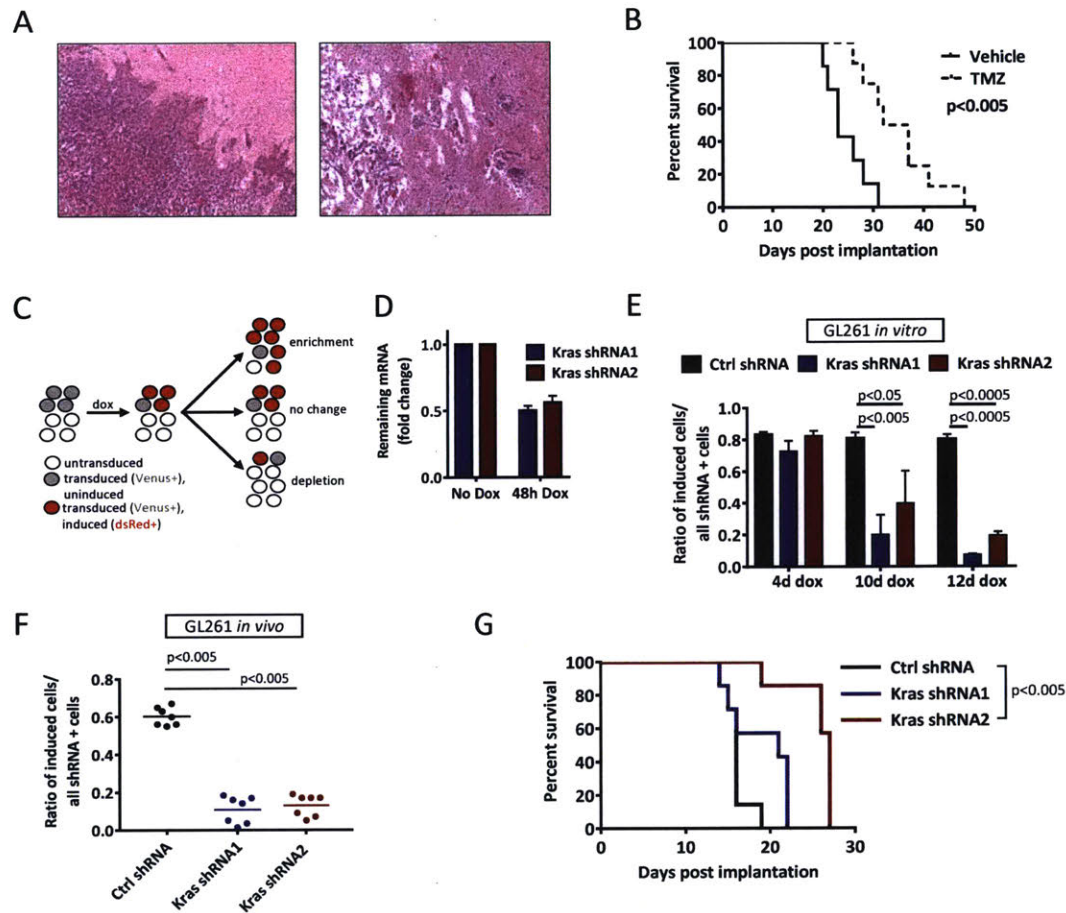
Figure 2.1. GL261 tumors resemble human tumors and respond to Kras depletion *in vivo*. (A) Tumors that form upon intracranial implantation of a syngeneic GL261 GBM cell line are invasive and highly vascularized, similar to human tumors. (B) Median survival of GL261 tumor-bearing animals (n=7) is 23 days, and is significantly extended to 35 days following treatment with TMZ (n=8, 50mg/kg, ip, at 16 days). P value determined by Log-Rank (Mantel-Cox) test. (C) Inducible TRMPVIR construct for miR30 based shRNA expression [325]. Transduced cells express constitutively active Venus (grey) and upon doxycycline induction, they also become positive for dsRed (red), indicating shRNA expression. The scheme outlines competition assays, performed by retroviral transduction of GL261 cells with this vector. Starting with a mixed population of cells where approximately half contained the vector, addition of doxycycline induces shRNA expression. Over time, shRNA expressing cells are either enriched, depleted, or undergo no change with respect to the starting population, depending on the identity of the shRNA. (D) Kras mRNA levels in GL261 cells containing one of two Kras targeting shRNAs, before and after doxycycline induction for 48h, as measured by qRT-PCR. n=3, and error bars represent SEM. (E) *In vitro* competition assay, showing proportion of Kras shRNA expressing cells over all transduced cells over time, compared to a Renilla shRNA control. n=3, and error bars represent SD, p value determined by Students t test. (F) *In vivo* competition assay, in which tumors were induced with doxycycline at 6 days post GL261 tumor cell implantation, to express either of the two Kras shRNAs or a Renilla shRNA control. n=7 for each condition, p value determined by Mann-Whitney test. (G) Survival of populations of mice presented in (F), p value determined by Log-Rank (Mantel-Cox) test.

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■ 2.3.2 Decreased Kras expression leads to measurable effects on tumor growth *in vivo*

We next sought to determine whether RNA interference by retroviral transduction of GL261 cells could lead to quantifiable changes in the composition of *in vivo* tumor cell populations. We used the inducible vector TRMPVIR [325] for shRNA expression (Figure 2.1C). Transduction of cells with TRMPVIR containing a specific shRNA results in the constitutive expression of the Venus fluorophore and rtTA3. Upon doxycycline treatment, rtTA3 binds the TRE promoter, inducing expression of the fluorophore dsRed and the shRNA. In vitro and *in vivo* cellular competition assays were performed to quantify effects of specific shRNAs at the cellular level. These assays started with a mixed population of cells, in which approximately half were transduced with the vector (and therefore expressed Venus), followed by addition of doxycycline to induce shRNA expression (resulting in Venus and dsRed double positive cells). After allowing for *in vitro* or *in vivo* growth, the proportion of shRNA expressing cells in the population was determined by flow cytometry. Depending on the identity of the particular shRNA, if the shRNA had beneficial, detrimental or neutral effects on cellular fitness, the proportion of shRNA expressing cells became enriched, depleted or unchanged, respectively (Figure 2.1C). To determine whether a single hairpin can have a measurable effect on tumor composition and growth, we targeted Kras, the known oncogenic driver in GL261 cells, hypothesizing that cells harboring Kras shRNAs would become depleted both *in vitro* and *in vivo*. Two different shRNAs resulted in decreased Kras mRNA expression 48 hours after doxycycline induction (Figure 2.1D). In vitro, knockdown with either of the two shRNAs caused a significant decrease in the ratio of induced shRNA expressing cells to all vector containing cells compared to a control shRNA (Figure 2.1E). To assess depletion of shKras expressing cells *in vivo*, we transplanted the same starting mixed populations of cells into mice, induced shRNA expression using doxycycline 6 days later, and isolated tumors from moribund animals. Flow cytometry analysis of the dissociated cell populations indicated a strong depletion of Kras shRNA containing cells with both of the two independent shRNAs, compared to a control shRNA (Figure 2.1F). Strikingly, although only a subset of tumor cells were impacted by Kras knockdown, this resulted in an appreciable lifespan extension (Figure 2.1G). Together, these results demonstrate that targeting the oncogenic driver Kras by knockdown in GL261 cells leads to a measurable effect on cellular fitness and animal survival. These data not only support the use of Kras shRNA as a positive control for depletion in

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the screen, but also show the efficacy of directly targeting Kras, which has important clinical consequences for a subset of GBM tumors that have deregulated Kras activity.

■ 2.3.3 In vivo GFP dilution experiments inform size of shRNA library

Transplantable solid tumor models are frequently subject to bottleneck and niche effects [33, 103], limiting their effective use for large pooled library *in vivo* screens. Thus, we sought to determine what proportion of intracranially implanted cells contribute to tumor formation in our system. The percentage of hairpins in a library that are represented in the tumor will reflect both the efficiency of engraftment of hairpin-containing cells and the proportion of cells that contribute to tumor growth [33, 201]. We assessed these considerations using an *in vivo* dilution experiment in which we intracranially transplanted GL261 cell populations containing different percentages of GFP positive cells and looked for the minimum percentage of GFP positive cells that can be detected in the final tumor (Figure 2.2A). In this manner, GFP expression acts as a surrogate for expression of a neutral shRNA. The number of cells that can be used for a screen in the brain is limited by the volume of cell suspension that can be in implanted without significantly increasing intracranial pressure and leakage from the site of injection. Since *in vivo* intracranial transplantations were performed with 500,000 cells, we tested two percentages, 0.2% and 0.02% GFP positive cells, that represent libraries in which each shRNA is expressed by 1000 or 100 cells, respectively. When the mice showed signs of high tumor burden, the tumors were isolated, dissociated into single cells and analyzed by flow cytometry. We reliably detected GFP positive cells in the final tumor cell population when starting with 0.2% but not 0.02% GFP positive cells (Figure 2.2B). This observation implies that if each shRNA in a pooled library is expressed in 1000 cells (with a lower limit of 100 cells), it can be detected in the final tumor cell population without being lost due to an inability of the cell to engraft or contribute to tumor formation, independent of the effect of the shRNA. This range is in line with values described throughout the literature [103, 201]. Under these conditions, detection of a specific shRNA would indicate an effect on cellular fitness. Therefore, we determined that a robust *in vivo* shRNA screen, where library representation is maintained throughout tumor progression, could consist of a pooled library of 500 to 5000 shRNAs in this model system.
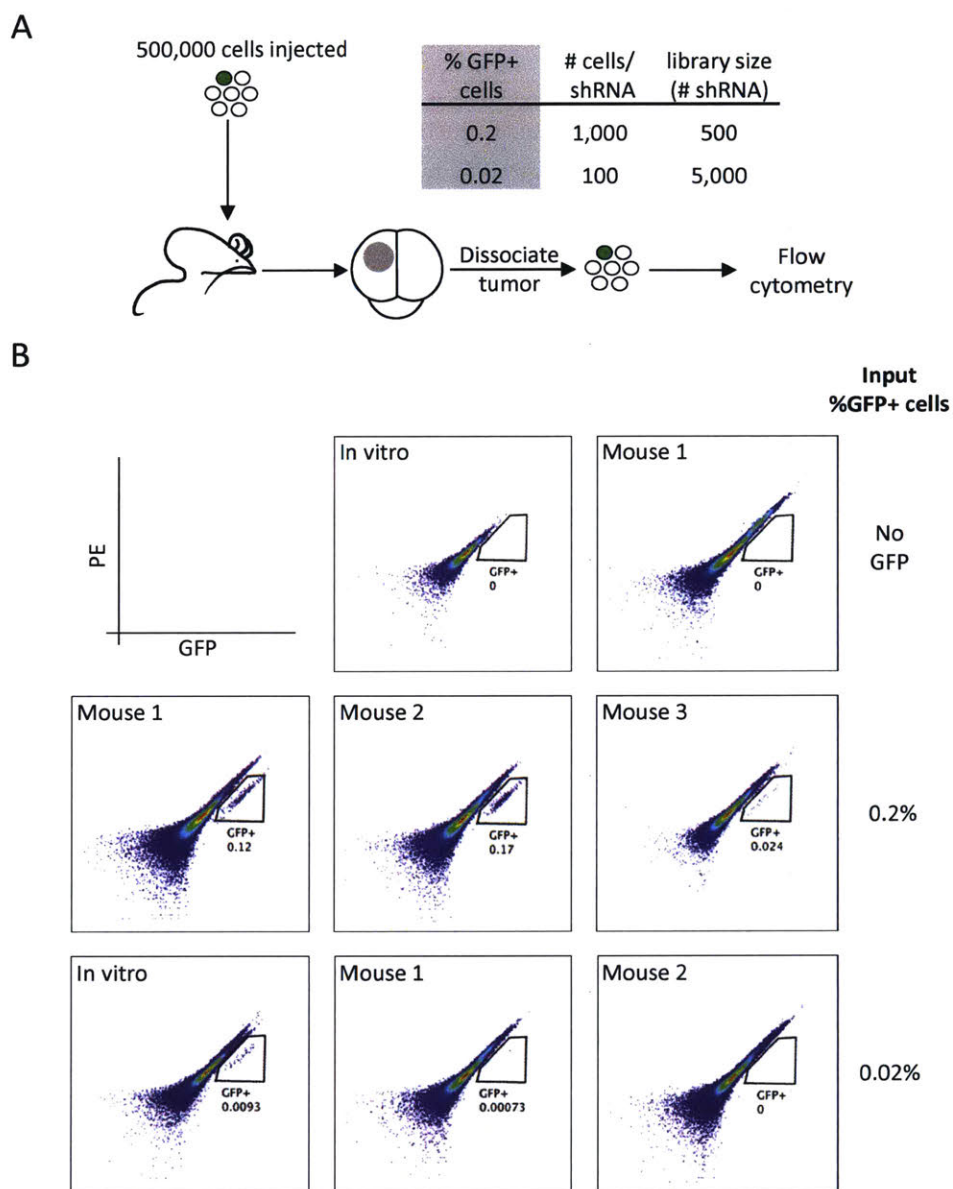
Figure 2.2. *In vivo* **dilution assay to determine the shRNA library size that can be reliably screened in GL261 system.** (A) Experimental design for GFP dilution assay to determine shRNA library size to be used for *in vivo* screen. Mixed populations of GL261 cells containing a set percentage of cells expressing a retroviral GFP construct (0.2% or 0.02%) were implanted intracranially, and the proportion of GFP positive cells was determined in the final tumors by flow cytometry. Table shows the number of cells / shRNA and the shRNA library size inferred from this experiment. (B) Flow cytometry plots showing the percentage of GFP positive cells recovered from the final tumors, depending on the percentage of GFP positive cells in the input population. Bottom left graph represents an *in vitro* control experiment to confirm that the lower percentage of GFP positive cells could in fact be detected by flow cytometry.

■ 2.3.4 GL261 GBM cells represent a reliable system for *in vitro* and *in vivo* shRNA screens

Due to the growing appreciation for the importance of epigenetics in GBM, we chose to use an shRNA library targeting epigenetic modulators to identify genes important for GBM tumor growth and response to TMZ. This high coverage library consisted of 2063 shRNAs targeting 313 genes (Figure 2.3A), the high coverage (approximately 6-10 shRNAs/gene) being critical for protecting against off target effects [251]. This number of shRNAs is well within the limits of our system, with each shRNA being expressed in approximately 250 cells. We spiked the library with the aforementioned Kras shRNAs at twice the concentration of the other shRNAs as positive controls for strong depletion. An input sample was obtained from GL261 cells following transduction and sorting for library expression, prior to intracranial transplantation (t=0). Following tumor development, mice were stratified into two groups and treated with vehicle or TMZ. In parallel, cells were cultured *in vitro* and treated similarly with vehicle or TMZ. When mice showed signs of morbidity, tumors were extracted and tumor cell DNA was sequenced. Survival analyses of the animals used for the screen showed that both the vehicle and TMZ treated cohorts behaved as expected for the model, with TMZ significantly extending lifespan (Figure 2.3B). The tumors that were used for sequencing were derived from animals that showed close to median survival. For each sample, more than 85% of the reads were mapped correctly to the shRNA library (Figure 2.3C). Furthermore, a high percentage of the original library shRNAs were recovered from each of the samples (Figure 2.3D). The input sample taken at t=0 contained 99% of the shRNAs from the original plasmid pool, indicating that library representation was preserved during viral packaging, transduction, sorting for library expression and *in vitro* cell expansion prior to start of the *in vitro* and *in vivo* screens. More importantly, an average of 90% of shRNAs from the original library were detected in all of the *in vivo* samples, even when requiring that each shRNA is called by 10 or more reads. Therefore, during tumor engraftment, growth, and TMZ treatment, very few shRNAs dropped down to undetectable levels. These results demonstrate that the GL261 system can be used for shRNA screens *in vitro*, and more importantly, *in vivo*, and successfully generate high quality data. Thus, we identified a rigorous screening platform even in the presence of the highly selective pressure applied by TMZ.

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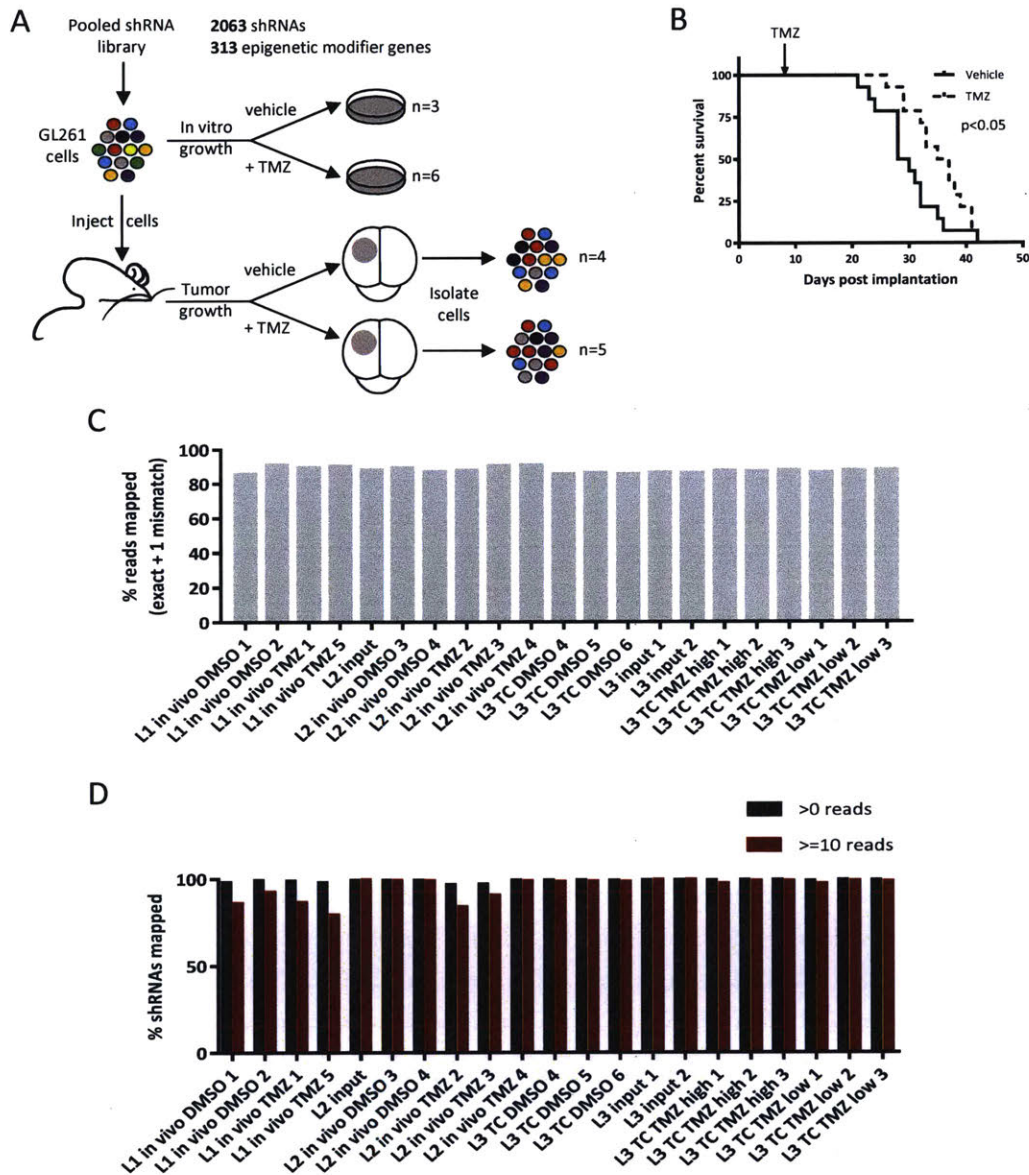
Figure 2.3. Parallel _in vitro_ and _in vivo_ shRNA screens demonstrating high quality data.
(A) Outline of screening strategy. Two TMZ concentrations were used, low ($7.5\mu M$) and high ($10\mu M$).
(B) Survival of animals cohorts used in screen, vehicle (n = 14), TMZ (n = 14) $p<0.05$ Log-Rank
(Mantel-Cox) test. DNA prepared from tumors obtained from a subset of the mice showing close
to median survival were used for the screen. (C) Percentage of reads that mapped correctly to the
shRNA library. (D) Percentage of the library shRNAs present in each of the samples, with stringency
of >0 reads or $>= 10$ reads. L1, L2, L3 represents lane 1, 2, or 3, according to how the samples were
sequenced.

■ 2.3.5 RNAi screen identifies PRMT5 as an important epigenetic modifier for GBM tumor growth

We used pairwise comparisons of the different screening conditions to extract high confidence candidates. First, to identify shRNAs depleted specifically in response to GBM tumor growth *in vivo* or cellular growth *in vitro*, we compared shRNAs depleted between the *in vivo* or *in vitro* vehicle treated conditions and the input sample at t=0 (Figure 2.4A). Next, shRNAs depleted specifically in response to TMZ treatment were detected by comparing vehicle to TMZ treatment in both *in vivo* and *in vitro* conditions (Figure 2.4B). In all four comparisons, we identified PRMT5 as one of the top shRNAs depleted, showing that knockdown of this gene is selected against during normal tumor cell growth as well as in response to therapy, both *in vitro* and *in vivo*. From the high coverage shRNA library, all 8 shRNAs targeting PRMT5 were depleted in the *in vitro* growth condition, and all but one depleted in the *in vivo* tumor growth condition (Figure 2.4C). The two control Kras shRNAs were selected against, as expected from earlier competition assay data. The results of the TMZ arm of the screen are presented and discussed separately in Chapter 3 of this thesis. We therefore chose to examine potential roles for PRMT5 in GBM growth.

■ 2.3.6 Loss of PRMT5 *in vitro* impairs GBM cellular fitness and is dependent on methyltransferase activity

To validate the results of the RNAi screen and to begin dissecting the role of PRMT5 on tumor growth, we first tested the effects of PRMT5 loss in several human GBM cell lines. We knocked down PRMT5 using three different inducible shRNAs in U-87 MG cells, resulting in loss of PRMT5 mRNA (Figure 2.5A) and protein expression, as well as the PRMT5-specific symmetric dimethylation of H4 arginine 3 (H4R3me2s, Figure 2.5C). Overexpression of shRNA resistant wild type PRMT5 cDNA in this setting restored PRMT5 mRNA (Figure 2.5B) and protein levels, together with H4R3me2s expression (Figure 2.5C). To test the functional effects of PRMT5 loss, we performed competition assays with mixed populations of U-87 MG cells containing a specific percentage of cells with PRMT5 shRNAs. Continued knockdown resulted in a strong depletion of shRNA expressing cells over time, compared to a control shRNA (Figure 2.5D). Similar results were observed with two other human GBM lines, U138 (Figure 2.5E) and A172 (Figure 2.5F) following 12 days of doxycycline-induced PRMT5 knockdown. Importantly,
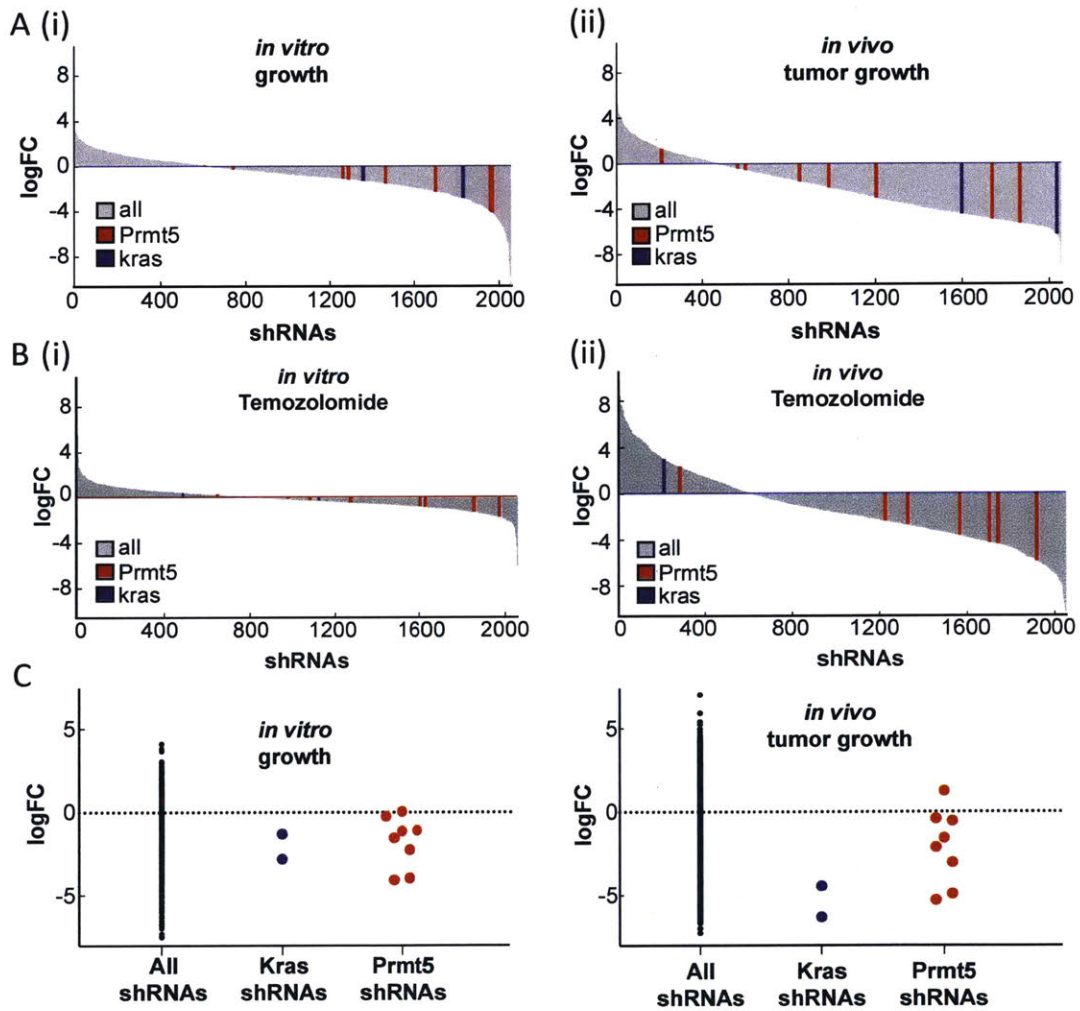
Figure 2.4. RNAi screen identifies PRMT5 as mediator of GBM growth *in vivo* and *in vitro*. (A) Waterfall plot of all shRNAs across *in vitro* (i) and *in vivo* (ii) samples comparing shRNAs enriched or depleted in response to vehicle vs input at t=0. (B) Waterfall plot of all shRNAs across *in vitro* (i) and *in vivo* (ii) samples comparing shRNAs enriched or depleted in response to TMZ. (C) Log fold change specifically for all 8 Prmt5 shRNAs and two control Kras shRNAs, vehicle treated condition *in vitro* (left) and *in vivo* (right). Prmt5 shRNAs are highlighted in red, and control Kras shRNAs in blue.
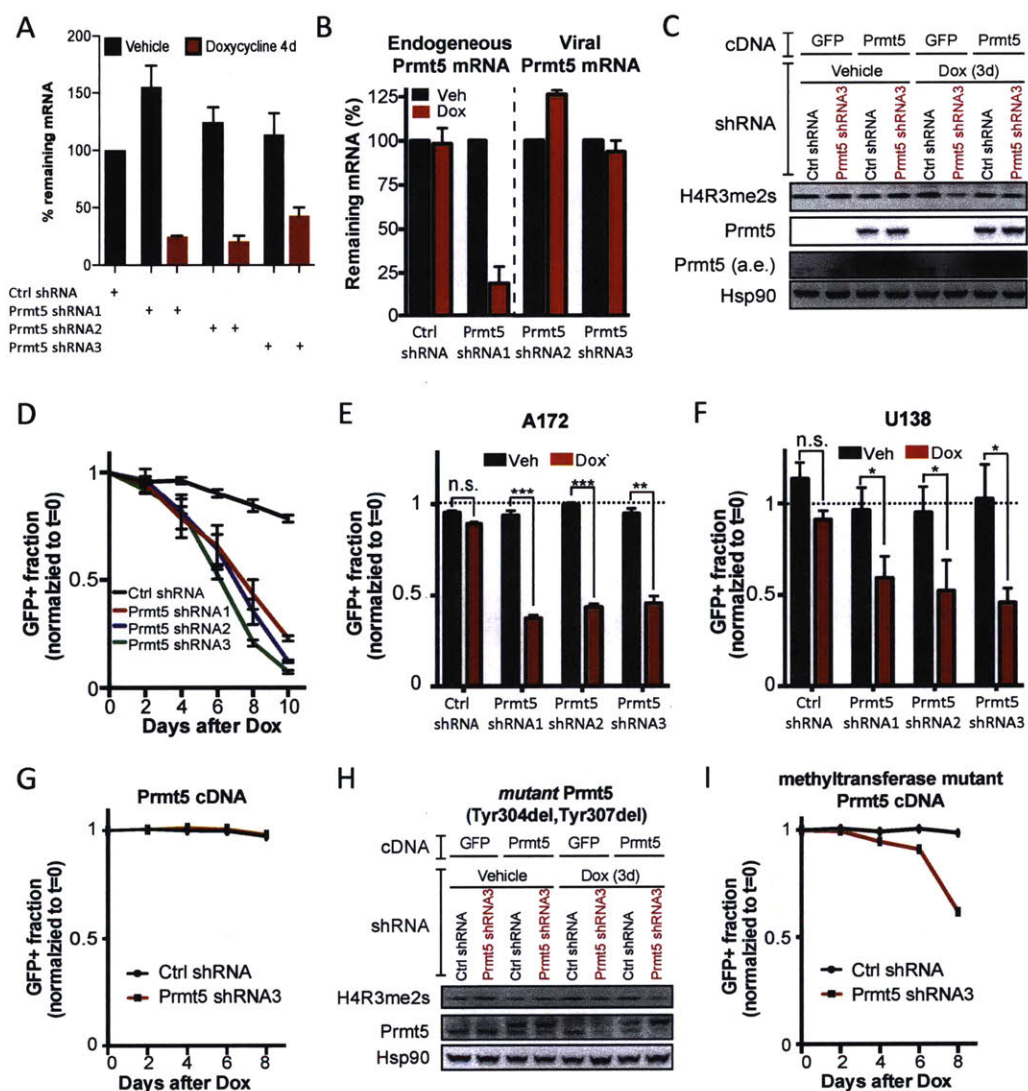
Figure 2.5. *In vitro* loss of PRMT5 impairs cellular fitness in a methyltransferase dependent manner. (A) PRMT5 knockdown in U-87 MG cells with three different inducible shRNAs or a control shRNA, as assessed by qRT-PCR. (B) qRT-PCR in U-87 MG cells showing PRMT5 mRNA levels upon knockdown, and co-expression of a PRMT5 cDNA. (C) Immunoblot of doxycycline- induced PRMT5 knockdown and wild type PRMT5 cDNA overexpression. (D) *In vitro* competition assays in U-87 MG cells containing PRMT5 or control shRNAs. (E) *In vitro* competition assay following 12 days of PRMT5 shRNA induction in A172 cells and (F) U138 cells. (G) *In vitro* competition assay in U-87 MG cells coexpressing PRMT5 shRNA and wild type PRMT5 cDNA. (H) Immunoblot showing expression of mutant form of PRMT5 cDNA containing deletion of Tyr304 and Tyr307. (I) *In vitro* competition assay in U-87 MG cells coexpressing PRMT5 shRNA and mutant cDNA.* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

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we showed that wild type PRMT5 cDNA rescued the depletion of PRMT5 shRNA expressing cells from a mixed population (Figure 2.5G). We then tested the requirement of PRMT5 methyltransferase activity for reversing the effects observed upon PRMT5 loss. Expression of a mutant form of PRMT5 containing deletions of residues that are important for methyltransferase activity (Tyr304 and Tyr307) [10] rescued PRMT5 protein expression, but not the loss of H4R3me2s modification noted upon PRMT5 knockdown (Figure 2.5H). Importantly, the mutant PRMT5 cDNA did not rescue the defect observed upon PRMT5 knockdown (Figure 2.5I). These results indicate that PRMT5 is important for the fitness of GBM cells across a number of human GBM cell lines. Furthermore, the methyltransferase activity of the enzyme is necessary for rescuing the anti-proliferative effects of PRMT5 loss.

■ 2.3.7 Inhibition of PRMT5 activity using a small molecule inhibitor impairs cell proliferation

The data presented thus far indicate that PRMT5 is important for GBM and suggest it as a therapeutic target in the disease. Fortunately, a highly specific small molecule inhibitor of PRMT5 has recently been published [44], so we tested whether the results of genetic loss of PRMT5 could be phenocopied by pharmacological inhibition [44]. Treatment of U-87 MG cells with EPZ015666 resulted in a time-dependent loss of SDMA, and more specifically, H4R3me2s, over 5 days (Figure 2.6A). Side by side comparisons showed that both PRMT5 shRNAs and PRMT5 inhibitor treatment achieved similar levels of SDMA depletion, but over different timescales (Figure 2.6B). Treatment of U-87 MG cells with EPZ015666 led to a time-dependent growth impairment (Figure 2.6C), with faster kinetics than using PRMT5 shRNAs. This effect was also observed to varying degrees in GL261, T98G and A172 cells (Figure 2.6C), indicating a range of sensitivities to the drug across different GBM cell lines. Taken together, these results demonstrate that inhibition of PRMT5 methyltransferase activity, whether by hairpin-mediated knockdown or by a small molecule, reduces cellular fitness in both murine and human GBM lines.
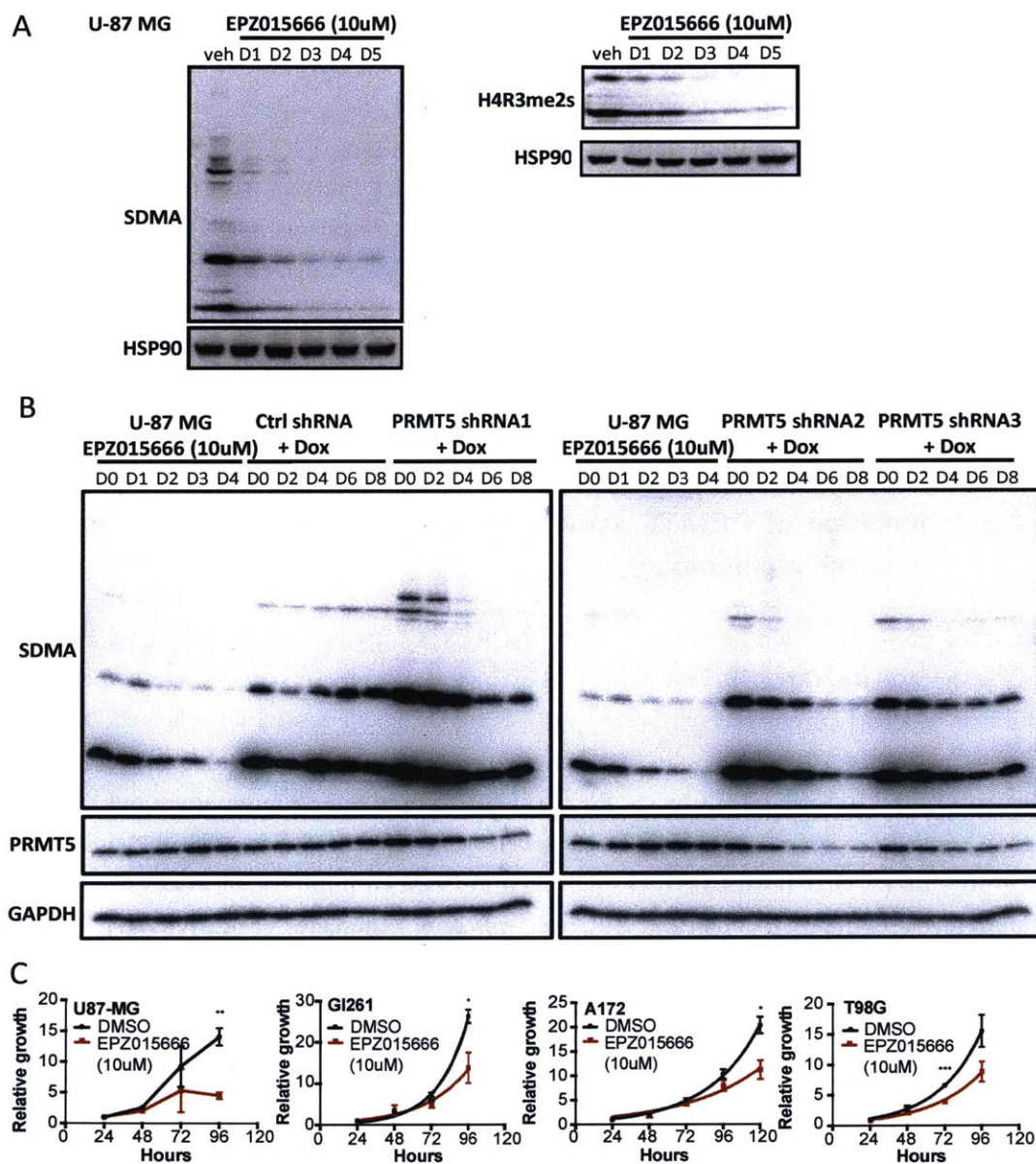
Figure 2.6. Inhibition of PRMT5 activity using a small molecule compound results in decreased proliferation across a variety of GBM cell lines. (A) Immunoblot of U-87 MG cells treated with the PRMT5-specific inhibitor EPZ015666 at a concentration of 10μM over 5 days. (B) Immunoblot of U-87 MG cells treated either with 10μM EPZ015666 over a period of 4 days or induced for shRNA-mediated knockdown of PRMT5 using doxycycline over 8 days. (C) Relative growth in four different human and murine GBM cell lines over time following treatment with 10μM EPZ015666. SDMA = symmetric dimethyl arginine. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.
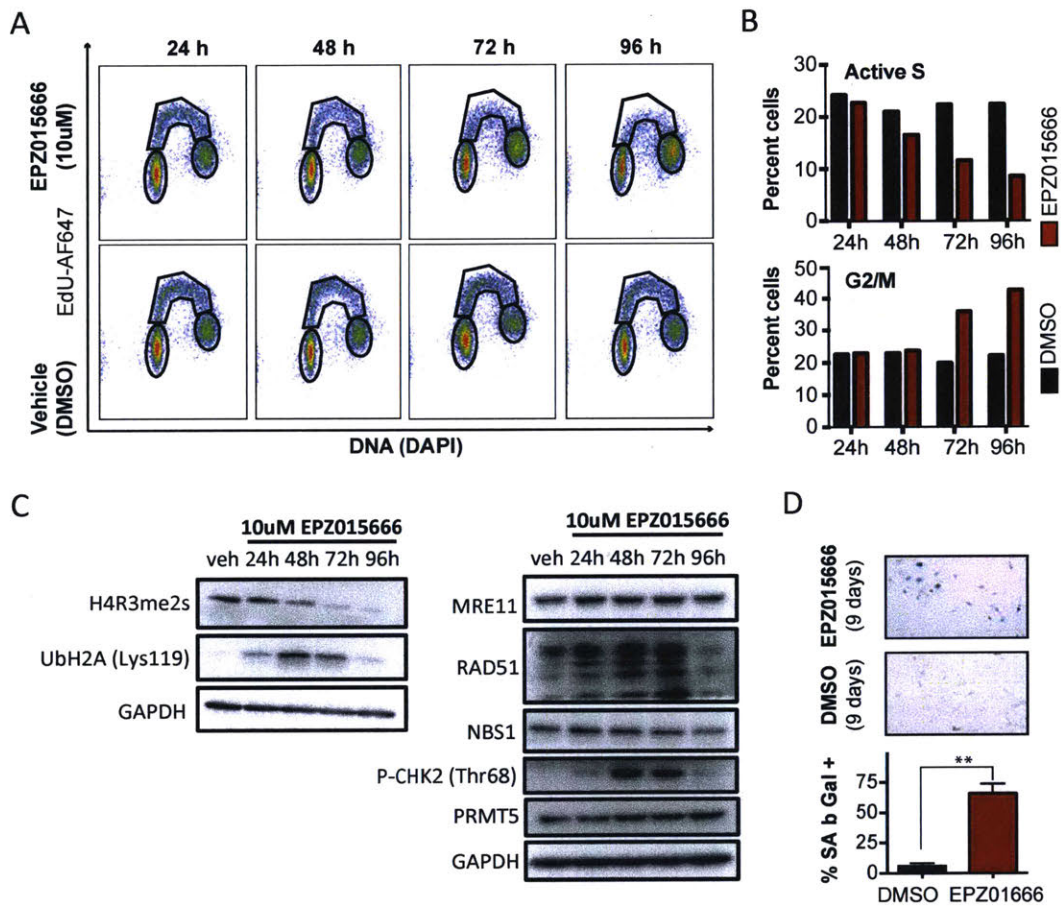
Figure 2.7. Inhibition of PRMT5 activity using EPZ015666 impairs cell cycle progression and promotes DNA damage response and senescence *in vitro*. (A) Flow cytometry-based cell cycle analysis in U-87 MG cells using EdU pulse and DAPI incorporation following treatment with EPZ015666 or vehicle. (B) Quantification of cell cycle analysis. (C) Immunoblot showing time course of various markers of the DNA damage response in U-87 MG cells following treatment with EPZ015666. (D) Senescence-associated -galactosidase staining of U-87 MG cells 9 days after treatment with EPZ015666. ** p<0.005

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■ 2.3.8 PRMT5 inhibition impairs cell cycle progression and promotes DNA damage response and senescence *in vitro*

To understand the effects of PRMT5 inhibition *in vitro*, we assessed cell cycle progression by flow cytometry in U-87 MG cells. After 24h-96h of treatment with EPZ015666 or a vehicle control, we used an EdU pulse to assess DNA replication, and DAPI incorporation to quantify DNA content (Figure 2.7A). The percent of cells undergoing active S phase decreased over time, while the percent of cells in G2/M increased following treatment with EPZ015666 compared to DMSO control (Figure 2.7B). We did not observe any increase in the apoptotic sub-G1 fraction upon PRMT5 inhibition. Several markers of DNA damage response pathways such as phosphorylated Chk2, Mre11, Rad51, NBS1, and UbH2A increased following EPZ015666, peaking 48 -72 hours after treatment (Figure 2.7C). Interestingly, we also noted that the morphology of U-87 MG cells changed following treatment, and they became senescent after 9 days (Figure 2.7D). Therefore, PRMT5 inhibition using a small molecule induces DNA damage responses and cell cycle arrest in the short term, and senescence in the long term.

■ 2.3.9 *In vivo* PRMT5 loss or inhibition impairs GBM tumor growth

Based on the proliferative impairment observed *in vitro*, we next tested whether genetic loss or pharmacological inhibition of PRMT5 *in vivo* would impact GBM tumor growth. Intracranial tumors were established in nude mice using mixed populations of human U-87 MG cells containing an inducible PRMT5 shRNA and a control shRNA. Mice were fed a continuous doxycycline diet to induce shRNA expression starting on day 5 following tumor cell implantation until they were euthanized due to tumor burden. We extracted the tumors and analyzed the ratio of induced shRNA-expressing cells to all vector containing cells by flow cytometry. In the tumors established with PRMT5 shRNAs, this ratio was significantly lower than in tumors established with control shRNAs (Figure 2.8A), indicating that PRMT5 knockdown cells were specifically at a disadvantage and selected against in the context of a mixed tumor cell population. Uninduced control tumors for both PRMT5 and control shRNAs were also analyzed, demonstrating that the retroviral vector did not induce expression of the shRNAs in the absence of doxycycline. These results indicate that loss of PRMT5 expression is detrimental in the tumor cell population, echoing the data from our *in vivo* shRNA screen where PRMT5 shRNA containing cells were selectively depleted. To address
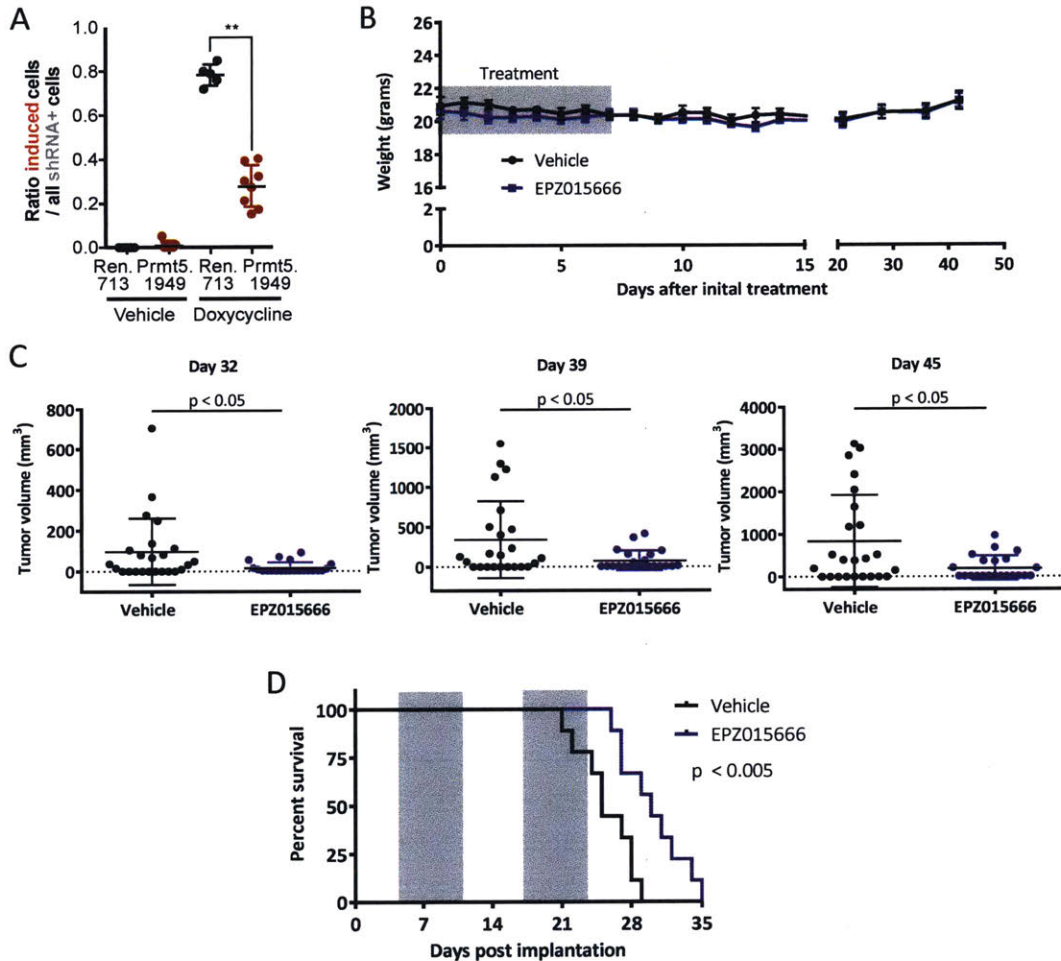
Figure 2.8. *In vivo* PRMT5 loss or inhibition impairs GBM tumor development and progression. (A) Mixed populations of U-87 MG cells were transplanted intracranially and induced with doxycycline starting at day 5 to express either a PRMT5 shRNA (n=8) or a control shRNA (n=5). Uninduced tumors are also shown, n=7 for PRMT5 shRNA condition, and n=5 for control shRNA condition. ** $p < 0.005$, as determined by Mann-Whitney test. (B) Non-tumor bearing C57BL/6 mice were dosed with 100mg/kg of EPZ015666 or vehicle twice a day by oral gavage, over a period of one week and their weights were recorded for a total of 6 weeks. (C) U-87 MG cells were transplanted subcutaneously and mice were treated with EPZ015666 at 100mg/kg, twice/day, between day 4-11 and day 33-40 post tumor cell implantation. Tumor volumes were measured on the indicated days. Vehicle treated condition n=24 (12 mice, both flanks), EPZ015666 treated condition n=22 (11 mice, both flanks). Error bars represent SD, p value determined by Mann-Whitney test. (D) Survival curve of mice injected intracranially with U-87 MG cells and treated with EPZ015666 (n=9) or vehicle (n=9). P value determined by Log-Rank (Mantel-Cox) test. Grey bars indicate treatment period. EPZ015666 was dosed at 100mg/kg, twice/day.

the clinical relevance of PRMT5 inhibition, we tested whether pharmacological inhibition of PRMT5 could impact survival of GBM-bearing animals. We first tested the toxicity of EPZ015666 by dosing non-tumor bearing C57BL/6 mice with 100mg/kg of drug twice a day by oral gavage, over a period of one week. The animals did not exhibit significant weight loss during the treatment or during a five-week follow-up period (Figure 2.8B), nor were their blood counts or liver enzymes abnormal compared to vehicle treated mice (data not shown). To begin addressing the therapeutic potential of PRMT5 inhibition, we tested whether administration of EPZ015666 would have an effect on tumor progression in a U-87 MG subcutaneous transplant model. Since the permeability of this small molecule across the blood brain barrier (BBB) has not previously been tested, this allowed us to measure the efficacy of PRMT5 inhibition in tumors in which exposure to the drug would be unobstructed. Experiments to address whether EPZ015666 is capable of crossing the BBB are currently ongoing. Treatment of mice bearing subcutaneous tumors with EPZ015666 for one week early during tumor development (4-11 days post tumor cell implantation) resulted in significantly smaller tumors by day 32 (Figure 2.8C). This effect was further enhanced after a second round of treatment late during tumor progression between day 33-40, resulting in dramatically smaller tumors in EPZ treated animals by day 39 and continuing post treatment, at day 45. This indicated that inhibition of PRMT5 had a measurable effect on tumor growth, and that PRMT5 was critical in the early stages of tumor development. Based on these results, we next tested the effect of PRMT5 inhibition in the context of an orthotopic tumor model, by using intracranial transplantation of U-87 MG cells. Treatment with EPZ015666 for 2 one week periods resulted in a significant lifespan extension (Figure 2.8D), indicating the potential therapeutic benefit of PRMT5 inhibition for the treatment of GBM tumors.

■ 2.3.10 Gene expression profiles reveal a shift in cell identity and GBM subclass following PRMT5 inhibition

To better understand the broad transcriptional changes that occur following PRMT5 inhibition, we performed poly(A)-selected RNAseq gene expression profiling of U-87 MG cells *in vitro* following treatment with EPZ015666. Based on the above results, we used the 72 hour timepoint, when SMDA was already strongly depleted, to capture the early transcriptional programs influencing proliferative impairment and appearance of the DNA damage response. Differential gene expression analysis revealed that 1625 (11.7%)

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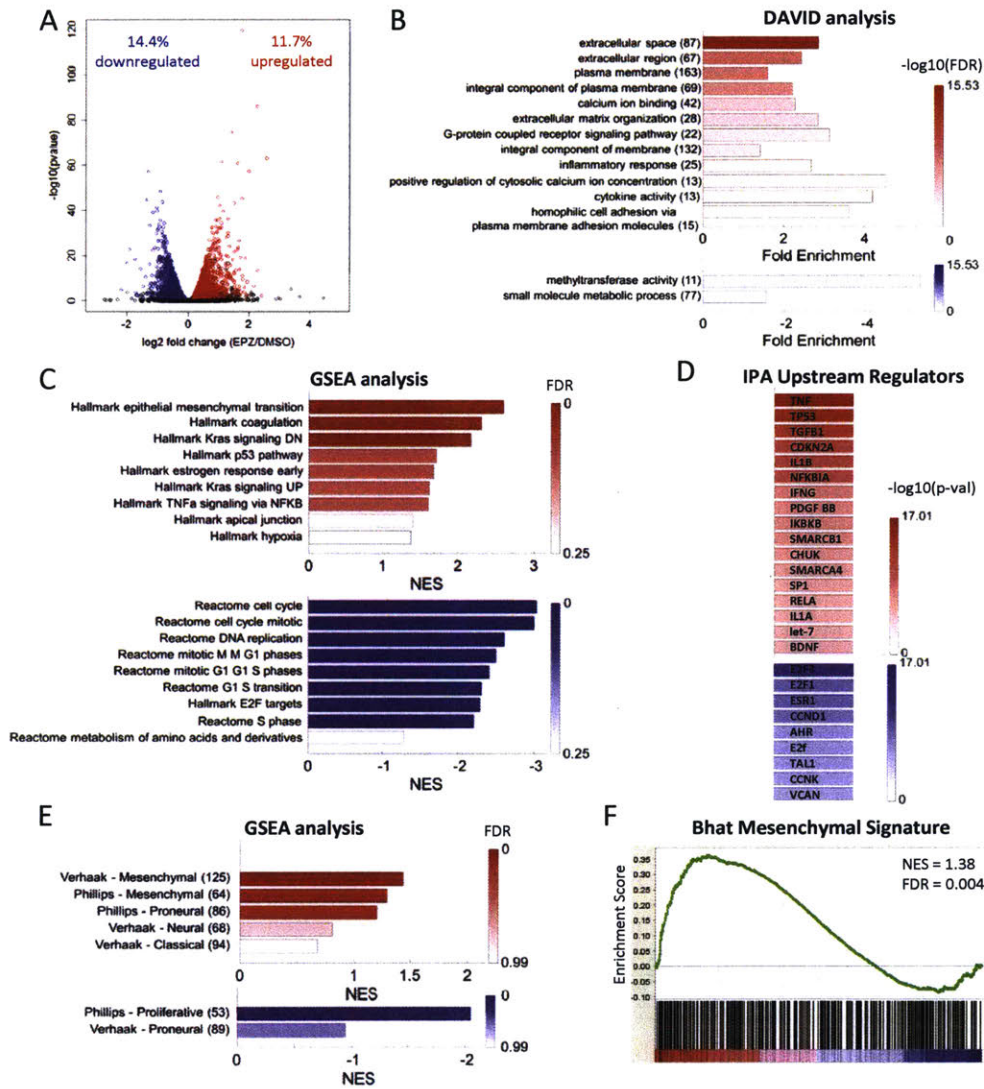
Figure 2.9. Functional pathway analyses of differentially expressed genes (DEGs) in EPZ015666 treated U-87 MG cells. (A) Volcano plot of all DEGs between EPZ015666 (n=3) and vehicle (n=3) treated U-87 MG in vitro samples, highlighting the genes that are significantly upregulated or downregulated with a p-adjusted < 0.05. (B) Gene ontology analysis of significantly upregulated or downregulated genes using DAVID. Gene set categories with FDR < 0.1 are displayed (C) Gene set enrichment (GSEA) analysis of significantly upregulated or downregulated genes. Gene sets from Hallmark and Reactome datasets with FDR < 0.25 are displayed. (D) Based on Ingenuity Pathway Analysis (IPA), list of predicted upstream regulators that can explain the observed gene expression changes. (E) GSEA analysis of different GBM subtypes enriched within the differentially expressed genes. NES = normalized enrichment score. Numbers in brackets indicate number of genes identified from that particular gene set. (F) GSEA enrichment plot showing enrichment of the Bhat et al. Mesenchymal signature [24] in the set of DEGs.

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and 2005 (14.4%) genes were significantly upregulated or downregulated, respectively (Figure 2.9A). Pathway analysis for enrichment of GO terms using DAVID [140, 141] uncovered upregulation of genes involved in extracellular matrix organization, cell-cell adhesion, inflammatory responses and cytokine signaling (Figure 2.9B). Meanwhile, downregulated genes were involved in methyltransferase activity and small molecule metabolism, pointing to the specific activity of EPZ015666. Gene set enrichment analysis (GSEA) [206, 268] revealed increased p53 and estrogen signaling, together with decreased cell cycle, DNA replication, and E2F targets (Figure 2.9C). Importantly, several upregulated gene sets pointed to pathways overlapping with the DAVID analysis, such as epithelial to mesenchymal transition (EMT), cell junctions, and TNF-α induced NF-κB signaling. To gain further insight into transcriptional regulators driving these pathways, we used Ingenuity Pathway Analysis (IPA), which predicted upstream regulators such as TNF, TGF-β, E2F1, and estrogen receptor ESR1 (Figure 2.9D). These three different methods of data analysis presented here indicate overlapping transcriptional programs deregulated by PRMT5 inhibition.

The pathway analyses point to increases in mesenchymal and immune signatures, and a concomitant decrease in progression through the cell cycle. Strikingly, these gene expression changes were reminiscent of transcriptional patterns defining different GBM subclassifications [232, 290]. Specifically, the mesenchymal GBM subtype is defined by mesenchymal and microglial gene signatures [232, 290]. Therefore, we asked whether PRMT5 inhibition could modulate transcriptional programs, resulting in an altered differentiation state. GSEA was used to compare the transcriptome data against gene signatures from each of the 3 and 4 GBM subtypes described in Phillips et al. and Verhaak et al., respectively [232, 290]. We noted an enrichment of genes characteristic of both Phillips and Verhaak mesenchymal subclasses, and a strong depletion of genes defining the Phillips proliferative subtype (Figure 2.9E). A more recent report has demonstrated that NF-κB signaling drives the transition of GBM tumors into a mesenchymal state [24], and defined a more complete list of genes associated with the mesenchymal signature. This gene set was predictive of poor response to radiation therapy in patients [24], and we found that it was significantly enriched in our differentially expressed genes (Figure 2.9F). These data suggest that treatment with a PRMT5 inhibitor drives a shift in the cell identity of GBM cells, driving them to express more mesenchymal features, with a concomitant decrease in a proliferative signature.

■ 2.3.11 Altered expression of splicing pathway components mediates resistance to EPZ015666

We wondered whether gene expression changes observed in response to PRMT5 inhibition could be indicative of transcriptional patterns reflecting relative sensitivity to EPZ015666. In light of our earlier results showing different levels of growth inhibition in various GBM cell lines in response to the drug, we hypothesized that gene expression changes over a large number of cell lines could be correlated with relative sensitivity or resistance to the inhibitor. Therefore, we screened 18 cell lines derived from different tumor tissues (Figure 2.10A), and tested the proliferation of these cells after 5 days over a wide range of drug concentrations and a vehicle control. The relative sensitivity or resistance to the drug over these concentrations was determined by activity area, according to procedures outlined by the Cancer Cell Line Encyclopedia (CCLE) [16]. This revealed a spectrum of sensitivity to EPZ015666 (Figure 2.10B), whereby the most sensitive cell line was U-87 MG, and the most resistant was SK-N-AS, a metastatic neuroblastoma line (Figure 2.10C).

Next, we used the publicly available transcriptome data from these 18 lines to interrogate the mediators of sensitivity versus resistance [16]. Specifically, correlations were calculated between activity area and gene expression values across all cell lines. GSEA analysis of the genes ranked by correlation revealed striking patterns, showing that classes of genes associated with RNA Pol II transcription and splicing were highly correlated with resistance to EPZ015666 (Figure 2.10D). Therefore, cells exhibiting higher expression levels of these genes are more likely to be resistant to PRMT5 inhibition by EPZ015666. These striking findings demonstrate that among the many cellular functions of PRMT5, it is specifically its role in splicing that largely mediates resistance to EPZ015666.

■ 2.3.12 Levels of detained introns with weak 5' splice sites are increased in response to EPZ015666

To gain a more in depth understanding of how alterations in the levels of splicing components contribute to the degree of sensitivity or resistance to EPZ015666, we queried changes in RNA splicing events in response to PRMT5 inhibitor using the RNAseq data from U-87 MG cells. We assessed the effect of PRMT5 inhibition on both constitutive and alternative splicing events by calculating the percent spliced in (PSI) values, repre-
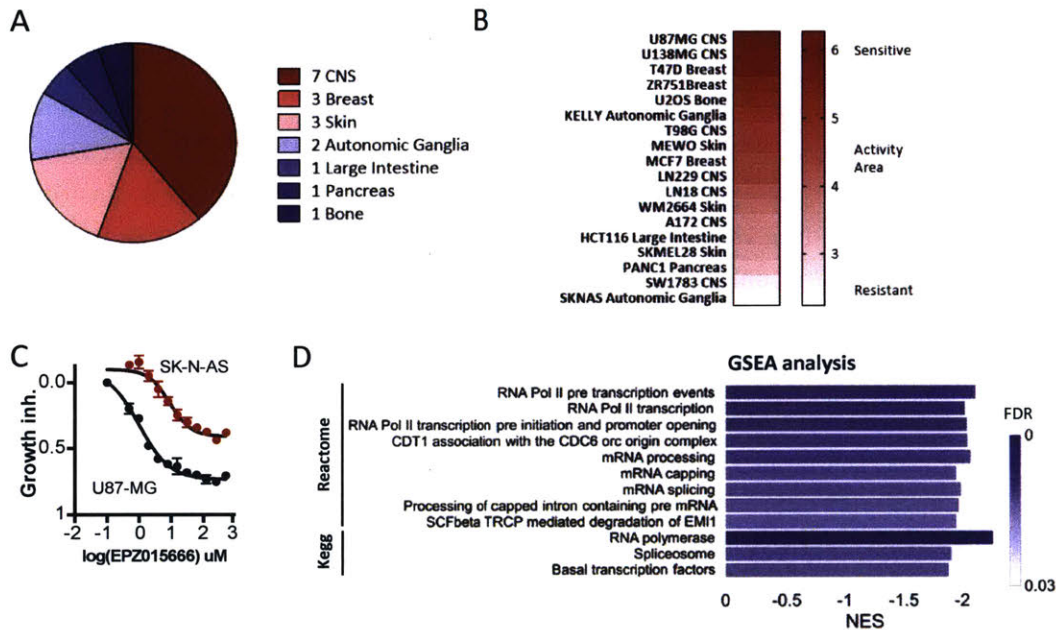
Figure 2.10. Cell line screen identifies splicing related pathways as critical mediators of resistance to EPZ015666. (A) Relative sensitivity to EPZ015666 was tested across 18 cell lines derived from different tumor tissues. Pie chart shows number of cell lines from each tissue type. (B) Ranking of all cell lines according to their relative sensitivity or resistance, as determined by activity area. Since 11 drug concentrations and one control concentration were tested, the activity area ranges from 0, representing no drug baseline, to 11, representing maximal activity at all concentrations. (C) Relative growth inhibition of most sensitive (U-87 MG) and most resistant (SK-N-AS) cell lines across a range of drug concentrations. (D) GSEA analysis showing that resistance to EPZ015666 is correlated with gene sets related to RNA transcription and splicing from the Kegg and Reactome datasets. NES = normalized enrichment score.

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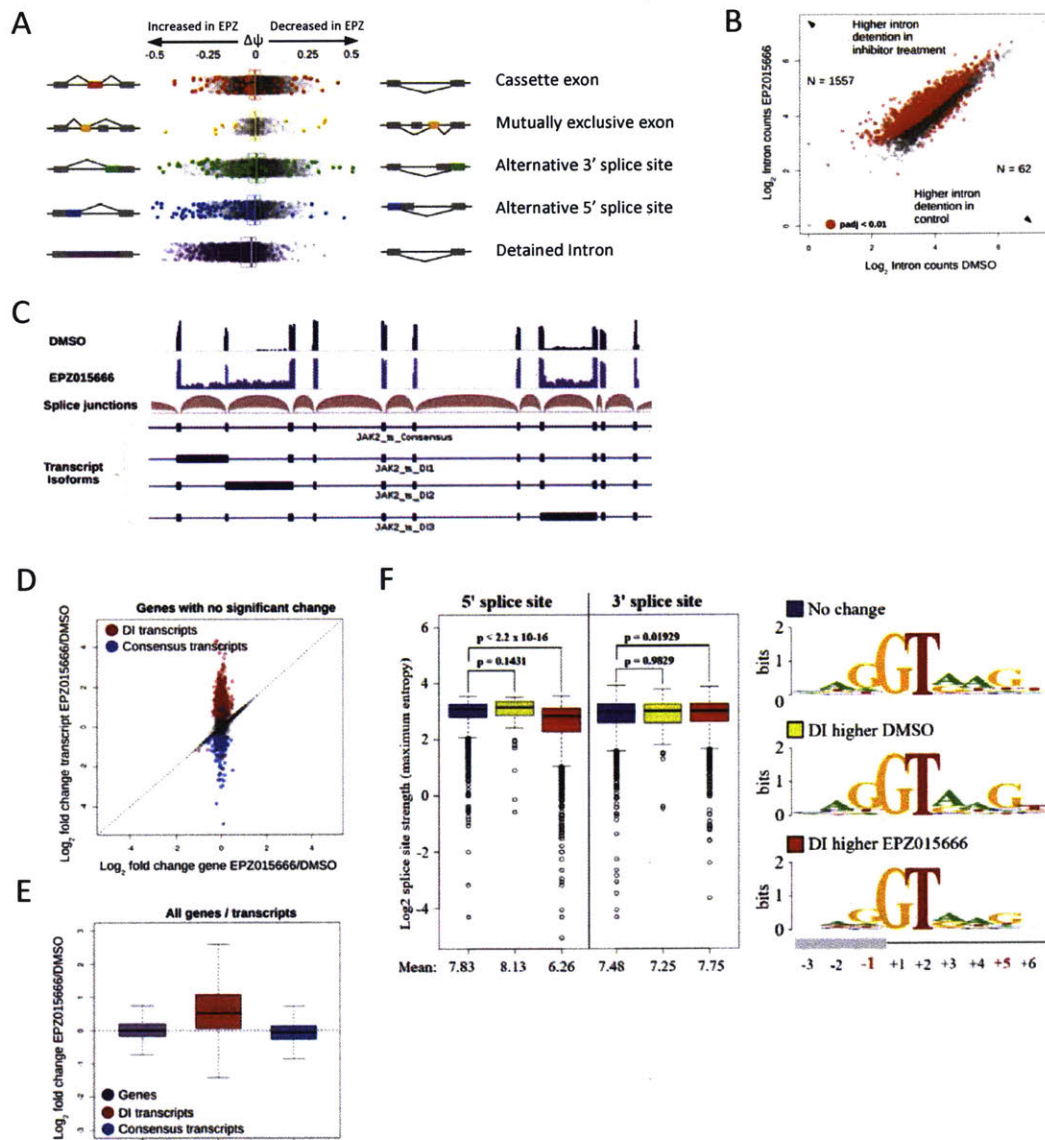
Figure 2.11. Analysis of differential RNA splicing identifies striking changes in detained introns. (A) Alterations in classes of constitutive and alternative splicing events in U-87 MG cells in response to EPZ015666. Values represent $\Delta\psi$, or change in percent spliced in, in EPZ015666 versus vehicle treated samples. (B) Plot showing number of detained introns in drug versus control treated samples. (C) Example showing increased DI-containing isoforms in JAK2 transcripts upon drug treatment. (D) A subset of genes showed no significant change in response to drug treatment at the total transcript level, but contained an increase in DI-containing transcripts and decrease in consensus transcripts in response to EPZ015666 treatment. (E) Levels of DI containing transcripts and levels of consensus transcripts, when normalizing to the gene level changes between drug and control treatments. (F) DIs whose expression is higher in EPZ015666 treated samples have weaker 5' splice sites.

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senting the percent of a particular isoform compared to all transcripts of that gene [79]. The difference between the EPZ015666 and vehicle treated conditions yielded a delta PSI value (Figure 2.11A). While there were few changes in cassette exons, mutually exclusive exons, alternative 5' and 3' splice sites, we discovered a strong increase in detained introns in the drug treated condition. Detained introns (DIs) have recently been described as a novel class of introns whose inclusion confines incompletely processed poly(A)-containing transcripts to the nucleus following transcription, preventing their translation into protein [29]. This subset of introns contrasts the more well-known class of retained introns. These completely processed mRNAs contain an intron that is retained within the message, and they are transported to the cytoplasm and translated. DI-containing transcripts remain in the nucleus, and in response to certain signals, the DI is either spliced out, resulting in a completely processed transcript that is then exported to the cytoplasm and translated, or degraded inside the nucleus. The discovery of DIs [29] is buttressed by numerous observations of delayed or post-transcriptional splicing of intronic sequences and speculations that these could be important for regulating gene expression [209, 280, 289]. The findings that such a specific set of splicing events is selectively affected by drug treatment indicates that PRMT5 inhibition results in a highly specific effect on splicing, as opposed to a global splicing defect. We identified 3801 DIs in total between control and drug treated cells. Of these, 2182 DIs were present in similar proportions under both conditions, showing that DI-containing transcripts represent a normal mechanism for regulating gene expression. Of the rest of the DIs, 1557 were more abundant specifically in drug treated samples, and only 62 were higher in control samples, representing 96% and 4% of a total of 1619 DIs that were differentially regulated in response to PRMT5 inhibition (Figure 2.11B). For example, DI containing transcripts of JAK2 were found to be present at higher levels in drug treated samples compared to controls (Figure 2.11C). This polarized shift in DI containing transcripts prompted us to speculate that PRMT5 is normally involved in pathways governing the splicing of detained introns as a mechanism of regulating gene expression. When looking at the total gene level, the presence of detained introns can mask the magnitude of changes in the levels of protein coding transcripts, therefore misrepresenting the alterations in gene expression upon drug treatment. For example, we identified a very specific class of 124 genes, whose levels of total mRNA did not change in response to drug treatment. Upon closer examination, these genes demonstrated an increase in DI-containing transcripts and a decrease in consensus protein coding transcripts in response to EPZ015666 treatment (Figure 2.11D). While not showing any

changes at the total mRNA level, these genes are expected to show decreased protein levels in response to drug treatment. Besides this special class, there was an overall increase in the levels of DI containing transcripts and an overall decrease in consensus transcripts, when normalizing to the gene level changes between drug and control treatments (Figure 2.11E). When probing further into the possible mechanisms behind the selective increase in DI events, we observed that DIs that were specifically increased in response to treatment with EPZ015666 had significantly weaker 5' splice sites, but not 3' splice sites (Figure 2.11F). Overall, these results point to a highly specific mechanism of PRMT5 activity in regulating splicing of detained introns with weak 5' splice sites, contributing to overall changes in gene expression.

■ 2.4 Discussion

In this work, we have presented the development and results of robust *in vivo* and *in vitro* shRNA screens in a transplantable GBM model that identifies the protein arginine methyltransferase PRMT5 as an important mediator of tumor growth. The use of genetic and pharmacologic tools supports the therapeutic potential of PRMT5 inhibition in GBM, and presents evidence for a possible role for PRMT5 in determining cell identity in these tumors. Furthermore, we characterize a role for PRMT5 in modulating gene expression by regulating a novel, distinct class of introns, and demonstrate the widespread dependence of PRMT5-related splicing functions across a variety of tumor cell lines. As part of developing and testing an appropriate system to use for *in vivo* screening, we established Kras as an oncogenic driver in the GL261 cell line, demonstrating that its expression is important for cell viability both *in vitro* and *in vivo*. While this carcinogen-induced GBM cell line undoubtedly contains numerous other mutations, the specific dependence on Kras demonstrates that targeting it by direct inhibition may be efficacious for the subset of GBM tumors that either contain Kras mutations or deregulations in upstream regulators.

We set up an orthotopic transplantation model using GL261 cells for loss of function *in vivo* screening. The results of initial pilot experiments demonstrated that this system meets three prerequisites for *in vivo* screening. First, we establish that tumor cells transplant with high efficiency and give rise to tumors resembling human GBM. Second, representation of at least a moderately large pooled shRNA library such as the one used in this study can be faithfully maintained during tumor cell engraftment,

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tumor growth, and even in the presence of a selective pressure such as that imposed by a chemotherapeutic agent. Lastly, shRNA depletion can be scored with high confidence and low variability across different samples. Therefore, this first screen also represents a proof of concept and establishes a robust model that can be used for further discovery screens or ones with a more targeted validation library. Consistent with previous reports [102, 241] this screen also demonstrates the value of *in vivo* RNAi depletion screens for identifying epigenetic regulators with roles in GBM.

The shRNA screen identified PRMT5 as being important for cellular fitness both *in vitro* and *in vivo*, independent of chemotherapeutic treatment with TMZ. Loss of PRMT5 activity *in vitro* using knockdown and a small molecule inhibitor impaired cellular proliferation in several GBM lines, and was dependent on methyltransferase activity, consistent with previously descried roles for PRMT5 as an oncogene [4, 55, 182, 312]. We found that PRMT5 inhibition impaired cell cycle progression, promoting DNA damage response and senescence *in vitro*. Interestingly, a recent report described a role for PRMT5 in mediating R loop resolution [323], offering a possible explanation for our observation of DNA damage induction upon PRMT5 inhibition. Although gene expression data following PRMT5 inhibitor treatment showed upregulated P53 signaling, consistent with other reports [23, 166, 185], we observe increased senescence as opposed to apoptosis. A recent study reported PTEN-dependent senescence in glioma neurospheres but not more differentiated adherent cultures. While the U-87 MG cell line is PTEN null, other possible explanations exist for the phenotype we observed [212]. It is possible that GBM cells are more likely to undergo senescence compared to apoptosis in response to PRMT5 inhibition, and this should be tested across a wider number of cell lines.

To our knowledge, this is the first report testing the effectiveness of the highly specific PRMT5 inhibitor EPZ015666 on survival in GBM-bearing animals. Our results demonstrating that administration of this drug can reduce tumor volume and increase survival are consistent with our data using genetic depletion of PRMT5 expression, as well as two previous reports [13, 312]. Together, these results support the importance of PRMT5 in GBM and advocate for further investigation into the efficacy of EPZ015666 in other animal models of GBM, as well as other tumor types known to overexpress PRMT5 [44].

Gene expression analyses identified that PRMT5 inhibition led to an enrichment for genes involved in extracellular matrix reorganization and EMT, as well as increased

NF-κB signaling and inflammatory responses, pointing to a mesenchymal GBM phenotype [24, 232, 290]. The mesenchymal subtype is associated with increased invasion and proliferation [41], and although we observed decreased proliferation in response to PRMT5 inhibition, invasiveness remains to be tested in functional assays. While several reports identify a positive role for PRMT5 in mediating TGF-B-dependent EMT in epithelial tumors [49, 147], the effects of PRMT5 on NF-κB-dependent transcription appear to be context dependent [4, 55]. For example, while Harris and colleagues report that PRMT5-mediated methylation of the NF-κB p65 subunit enhances CXCL10 secretion in endothelial cells [127, 128], PRMT5 depletion in GBM cells resulted in increased expression of CXCL10 and CXCL11 [312]. Given the clinical relevance of the mesenchymal subtype [24, 41], these results warrant further investigation *in vitro* and in other GBM models *in vivo*. Using a diverse panel of cancer cell lines, we determined that resistance to EPZ015666 is correlated with increased expression of the spliceosomal machinery. Although the causality of this association remains to be tested, for example by knockdown of different spliceosomal components to see whether drug sensitivity can be restored, these results offer important insight into the pathways that mediate resistance to EPZ015666. This may not only lead to a predictive signature of tumors that are more or less likely to benefit from PRMT5 inhibition, but it opens the door to exploring synthetic lethality and combination therapies for exploiting this dependence.

In studying splicing changes associated with PRMT5 inhibition, we achieved four main results. First, we find that PRMT5 does not act to indiscriminately control splicing capacity. Instead, it specifically regulates a particular subset of DI-containing transcripts. Conversely, not all DIs are regulated by PRMT5, indicating that there are additional mediators of DI inclusion, such as Clk kinases and others [29]. Second, PRMT5 inhibition favors the inclusion of DIs characterized by weak 5' splice sites, and the exclusion of others, which are increased in the vehicle condition and remain to be characterized. This infers that PRMT5 normally favors the splicing of elements with weak 5' splice sites, as has been previously reported [23, 71, 166, 243]. These results point to a highly specific role for PRMT5 in the splicing process, perhaps in regulating 5' splice site selection, via U1snRNP-specific proteins which are also PRMT5 targets [51, 131]. As DIs were previously found to be both constitutively and alternatively spliced [29], it will be important to determine what proportions of PRMT5-regulated DIs fall into each of these categories. Third, we only see a small number of alternative splicing events that do not contain DIs. Previous studies have reported changes

in retained introns as well as skipped exons upon PRMT5 depletion [23, 166], and we believe that most of these events represent DIs, with the exception of a small subset. Lastly, we find that overall, DIs increase in response to PRMT5 inhibition, and coding transcripts decrease. This observation implies that depending on the balance at the level of an individual gene, this can result either in an upregulation of total mRNA levels, a downregulation or no change. However, viewing these alterations at a total gene level masks the fine-tuning that DIs offer, as only changes in the coding transcript translate into changes at the protein levels. Therefore, in poly(A) selected RNAseq, DIs likely conceal gene expression changes at the protein level. It will be important to see how these different categories segregate in response to treatment and whether there are enrichments for specific sets of genes or pathways that are specifically regulated by differential DI inclusion in response to PRMT5 inhibition. Critically, not all gene expression changes in response to PRMT5 inhibition can be attributed to DIs, indicating that PRMT5 uses splicing as part of a larger program of regulation of gene expression. This may include transcriptional changes through modulation of chromatin structure, transcription, or direct impact on signaling effectors. Further exploration of these ideas may point to PRMT5 as a regulator of post-transcriptional splicing and may even uncover roles in co-transcriptional splicing, as has been described for other epigenetic regulators [193, 256]. Introns are the most poorly understood class of splicing elements and they are found to be differentially regulated across a variety of cancers [79], so these findings may position PRMT5 as playing a central role in tumorigenesis by integrating a diverse set of mechanisms for modulating gene expression.

Taken together, our data demonstrate that PRMT5 can be successfully targeted by a small molecule inhibitor, leading to measurable outcomes in GBM-bearing animals. PRMT5 appears to drive gene expression changes in part by modulating splicing of a specific set of intronic elements, and the contributions of this function to tumorigenesis will continue to be explored. These data raise numerous exciting questions about the oncogenic functions of this methyltransferase and demonstrate implications across a broad range of tumor cell types.

■ 2.5 Materials and Methods

Animal Studies

C57BL/6 female mice aged 6-8 weeks were purchased from Jackson Laboratories (Bar Harbor, MA). Athymic female mice (NCr nu/nu strain) aged 6-8 weeks were purchased from Taconic Laboratories (Rensselaer, NY). Intracranial transplantations were performed by injecting 500,000 cells in sterile PBS into the left hemisphere, approximately 2mm to the left of the bregma along the coronal suture. Mice were monitored for signs of disease based on predefined criteria. Subcutaneous transplantations were performed by injecting 1×10^6 cells in sterile PBS into both flanks. Subcutaneous tumor volumes were measured on the indicated days using a precision caliper. All animal studies were performed in accordance with MIT Committee on Animal Care protocol approval.

Reagents

Temozolomide (TMZ) was purchased from Sigma (T2577) dissolved in DMSO. For *in vivo* experiments, TMZ was further diluted in sterile 0.9% sodium chloride. EPZ015666 was purchased from DC Chemicals (DC8012) and resuspended in DMSO for *in vitro* experiments. For *in vivo* experiments, EPZ015666 was dissolved in 0.5% methylcellulose in water. Doxycycline was purchased from Clonetech (631311) and dissolved in water. *In vitro*, cell lines were treated with $1\mu g/mL$ to $10\mu g/mL$. *In vivo*, doxycycline was either administered in the drinking water, dissolved in 2% sucrose water to 1mg/mL, or in the form of doxycycline containing food pellets, purchased from Harlan Teklad (TD.01306), for the indicated time periods.

Competition assays

Cells were infected *in vitro* with the indicated vectors and sorted by fluorescence activated cell sorting (FACS) to generate pure populations of transduced cells. These populations were then mixed with untransduced cells to create mixed populations. After 96 hours, the proportion of GFP expressing cells was assessed by flow cytometry. For *in vivo* competition assays, a mixed population of 500,000 cells, same as ones used for *in vitro* assays, were transplanted intracranially into C57BL/6 mice. Animals were monitored and when they showed signs of high tumor burden according to predefined

criteria, they were euthanized and the brains were extracted. The tumors were excised and dissociated into a single cell suspension using a Brain Tumor Dissociation Kit from Miltenyi Biotech (130-095-942) and using a gentleMACS Dissociator (Miltenyi Biotech) according to manufacturer recommendations. Cells were analyzed by flow cytometry to determine the percentage of GFP expressing cells using a BD LSR II machine and analyzed using FlowJo software.

Vectors used

The constitutive retroviral vector MSCV/LTR/miR30/SV40/GFP (MLS) and the inducible retrovial vector TRMPVIR (Addgene 27994) were used. The constitutive retroviral vector pRSF91-GFP-miRE, a kind gift from Adrian Schwarzer and Axel Schambach (MH Hannover, Germany) and the inducible retroviral vector Tre-dsRed-miRE-PGK-Venus-IRES-rtTA3 (RTREVIR), a kind gift from Johannes Zuber [87] (IMP Vienna, Austria), were also used. pLJM1-EGFP was a gift from David Sabatini [242] (Addgene 19319). The codon-optimized PRMT5 cDNA was synthesized using Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific). PCR amplification was performed with the following primers: forward 5'- CGTCAGATCCGCTA-GATGGCGGCTATG, reverse 5'- TCGAGGTCGAGAATTCAGGCCGATGGTG and cloned into pLJM1-GFP by Gibson assembly [106]. Site directed mutagenesis was used to create the mutant PRMT5 DNA (Tyr304del, Tyr307del) using the following primers: PCR reaction 1: forward 5'- CGTCAGATCCGCTAGATGGCGGCTATG, reverse 5'- GCTCTGCAGATCCTCGCCCTTAGC; PCR reaction 2: forward 5'- GC-TAAGGGCGAGGATCTGCAGAGC, reverse 5'- TCGAGGTCGAGAATTCAGGCC-GATGGTGT.

shRNAs and primers used

Name of shRNA	Organism	mRNA target sequence
PRMT5 shRNA1 - PRMT5.163	Human	5'-CCCCGAAATAGCTGACACACTA
PRMT5 shRNA2 - PRMT5.1949	Human	5'-ACAGCACCAGTCTGTTCTGCTA
PRMT5 shRNA3 - PRMT5.671	Human	5'-TTCCGGACTTTGTGTGACTATA
KRAS shRNA1 - KRAS.1442	Mouse	5'-GCAGACCCAGTATGAAATAGTA
KRAS shRNA2 - KRAS.923	Mouse	5'-TGGAAACCTTCTTTTTTCTAAG
Ctrl shRNA - REN.713	Renilla luciferase	5'-TAGGAATTATAATGCTTATCTA

Primer	Organism	Primer sequence
GAPDH forward	Mouse	5'-AGAACATCATCCCTGCATCC
GAPDH reverse	Mouse	5'-CACATTGGGGGTAGGAACAC
KRAS forward	Mouse	5'-GCCTGCTGAAAATGACTGAGT
KRAS reverse	Mouse	5'-TTAGCTGTATCGTCAAGGCG
PRMT5 forward	Mouse	5'-CGCTCTGGTTGTTAAGGCGA
PRMT5 reverse	Mouse	5'-AGAGGAAATCAAACCCCTGCTT
GAPDH forward	Human	5'-GACAGTCAGCCGCATCTTCT
GAPDH reverse	Human	5'-GCGCCCAATACGACCAAATC
PRMT5 forward	Human	5'-CGCTCTGGTTGTTAAGGCGA
PRMT5 reverse	Human	5'-AGAGGAAATCAAACCCCTGCTT
PRMT5 codon optimized forward	Human	5'-GAAACAAACGTGCAGGTGCT
PRMT5 codon optimized reverse	Human	5'-ACGATGATGTCGGCCTTCTC

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In vitro and *in vivo* shRNA screen

A pooled shRNA library targeting 311 epigenetic genes using 2059 shRNAs was obtained from TansOMIC Technologies (Huntsville, AL). This library was subcloned from the original backbone MSCV/LTR/mIR30/PGK/Puromycin/IRES/GFP (pMLP) retroviral vector into the MSCV/LTR/mIR30/SV40/GFP (MLS) retroviral vector. Two shRNAs targeting Kras and two shRNAs targeting Msh2, in the MLS retroviral vector, were incorporated into the library at 2x the concentration of the other shRNAs, as controls, resulting in a total of 2063 shRNAs. GL261 cells were infected with the pooled shRNA library at a low infection efficiency in order to obtain singly transduced cells and prevent the possibility of multiple infections with different shRNAs in one cell. Cells were sorted for GFP by flow cytometry and expanded *in vitro* to obtain sufficient numbers of cells for injections. On day 0, cells were split into three samples: one for the *in vitro* arm of the screen, one for *in vivo* transplantation, and one to be taken as the input sample for the screen. *In vitro*, cells were split into 9 groups, 3x treated with DMSO vehicle as controls, 3x treated with low dose TMZ (7.5μM), and 3x treated with high dose TMZ (10μM). Cells were passaged every few days, maintaining no fewer than 500,000-1,000,000 cells each time, in order to preserve library representation without bottlenecking. *In vivo*, mice were intracranially implanted with 500,000 cells on day 0. On day 8, mice were split into two groups consisting of 14 animals each, treated with vehicle or TMZ (50mg/kg) by a one-time intraperitoneal injection. *In vitro* cultures were treated with vehicle or TMZ at the above specified concentration on the same day as *in vivo* treatment. Mice were monitored and euthanized when they developed signs of high tumor burden. When approximately half of the mice had been euthanized, *in vitro* samples from the 9 conditions were also harvested. Brains were harvested from the animals and tumors were carefully extracted, ensuring that minimal normal tissue was included. Tumor tissues were snap frozen in liquid nitrogen and genomic DNA from tumors, *in vitro* samples, as well as the input sample, were extracted using phenol-choloform. *In vivo* samples taken from mice with close to median survival (5 for TMZ treated, 4 for vehicle treated), were used for further analysis. shRNAs were amplified from genomic DNA using HotStarTaq DNA Polymerase (Qiagen, 203205). The following primers were used:

Forward: - 5'-CAAGCAGAAGACGGCATACGAGATNNNNNN gttgcctgcacatcttggaaa-cacttgct

Reverse: - 5'-AATGATACGGCGACCACCGAGATCTACAC taaagtagccccttgaattcc-

gaggcagtaggc

Index read primer: - 5' -TCCcagcaagtgtttccaagatgtgcaggcaac

Hairpin primer:5' - TAGCCCCTTGAATTCCGAGGCAGTAGGCA

Six nucleotide barcodes (NNNNNN) were used for multiplexing. Samples were sequenced on a HiSeq system (Illumina). From the one input sample genomic DNA, two replicates were created by amplifying shRNA sequences separately, and sequencing on separate lanes of the Illumina HiSeq. *In vivo* samples 'were sequenced on two separate lanes, while all *in vitro* samples were sequenced on one lane. Sequences were mapped to the shRNA library using Bowtie [178]. Raw read counts for every hairpin were used as input for hairpin and combined gene level data analysis using shRNA-seq via an online Galaxy workflow [64].

Immunobloting

Cells were harvested by scraping on ice and lysed with RIPA buffer. H4R3me2s (Abcam, ab5823), PRMT5 (Cell signaling, 2252S), GAPDH (Santa Cruz sc365062), actin (Santa Cruz, sc1616), symmetric dimethylarginine SDMA (Cell signaling, 13222), MRE11 (Novus, NB100-142), RAD51 (Santa Cruz, sc6862), NBS1 (Novus, NB100-143), P-Thr68-CHK2 (Cell Signaling, 2661S), Ub-Lys119-H2A (Cell Signaling, 8240S), HSP90 (BD, 610418, Clone 68). Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used for detection and imaged with an ImageQuant LAS4000 imager (GE) or by autoradiography film.

Histological analysis

Tissues were fixed in 10% formalin overnight, transferred to 70%ethanol and embedded in paraffin. Tissue sections of 5μM sections were stained with hematoxolyn and counterstained with eosin.

Cell cycle analysis

EdU (ThermoFisher Scientific, A10044), was dissolved in DMSO and added to cells at a concentration of 10μM for a period of 30 minutes at 37C. Cells were then harvested with 0.25% Trypsin/EDTA, washed with ice-cold PBS and fixed with ice-cold 100% ethanol. Cells were permeabilized by resuspending in ice-cold 0.25% TritonX100 in PBS for 15

minutes at 4C. They were then rinsed with 1% BSA in PBS and stained with a Click-iT Cell Reaction Kit (ThermoFisher Scientific, C10269) according to manufacturer instructions. Cells were once again washed with ice-cold 0.25% TritonX100 in PBS, and stained with 1μg/ml DAPI (ThermoFisher Scientific, D1306) at room temperature for 15 minutes, protected from light. Flow cytometry analysis was performed using either BD Fortessa or BD LSR-II HTS machines, and data was analyzed using FlowJo analysis software.

RNAseq

U-87 MG cells were treated with EPZ015666 at a concentration of 10μM for a period of 72h. Total RNA was isolated using the NuceloSpin RNA kit (Machery-Nagel) and tested for quality using the Agilent 2100 Bioanalyzer (Agilent). PolyA mRNA enrichment, cDNA synthesis, and library generation were carried out using the TruSeq RNA library preparation kit (Illumina) according to the manufacturer instructions. Sequencing was performed using 75nt paired end reads with a NextSeq sequencer (Illumina). Raw RNA-seq reads were mapped to Hg19 with genome annotation based on Gencode v19 using STAR aligner version 2.4.1 [76]. Custom Python scripts were used to map splice junctions as well as alternative and constitutive splicing classifications. MISO [161] was used to quantify differences in alternative splicing events. Reads were mapped using Bowtie 1.0.1 [178] and used to identify detained introns. Differences in detained intron splicing efficiency were determined using DEXSeq [8]. Relative splice site strength was calculated using MaxEnt [316] and the splice site motifs were determined using Weblogo [61]. For more details please see [29].

Functional pathway analyses

DAVID: Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 was used for analysis [140, 141]. Lists of differentially expressed genes, either significantly upregulated or significantly downregulated, using a p adj<0.05, were tested against all expressed genes in the gene expression dataset.

GSEA: We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB) (Subramanian, Tamayo, et al. 2005; Mootha et al., 2003; http://www.broad.mit.edu/gsea/). Analyses were performed using the pre-ranked tool and differentially expressed genes with a p adj<0.05. Gene

set enrichment scores were calculated for the following gene sets: Hallmark [183], CP:KEGG: KEGG gene sets [158, 159] (http://www.genome.jp/kegg/pathway.html); CP:REACTOME: Reactome gene sets [60, 84] (http://www.reactome.org/). Custom analyses were performed by comparing all expressed genes to gene expression signatures representing different GBM subtypes [24, 232, 290].

IPA: Data were analyzed through the use of QIAGENs Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

qRT-PCR

Total RNA was extracted using the NucleoSpin RNA kit (Machery-Nagel) and cDNA was generated using PrimeScript RT kit with gDNA Eraser (Takara). Reactions were performed using the Fast SYBR Green PCR Mastermix (Applied Biosystems) and a StepOnePlus Real-Time PCR System (Applied Biosystems) using StepOne Software v2.2.2 (Applied Biosystems). GAPDH was used an internal control.

Cell proliferation

To assess *in vitro* cell proliferation, cells were stained with the CellTrace Far Red Cell Proliferation Kit (Thermo Fisher, C34564) according to manufacturer instructions. Briefly, cells were resuspended with the Far Red staining solution for 20 minutes at 37C, washed, and then incubated with EPZ015666 for the indicated number of days. Cells were the trypsinized, analyzed by flow cytometry and the mean fluorescence at 24 hour post labeling was used as a baseline. The relative growth was then calculated as the reciprocal of the mean fluorescence.

Senescence associated beta-galactosidase staining

U87-MG cells were plated and treated with 10μM EPZ015666 24 hours later. At 9 days post-treatment, cells were stained for senescence-associated β-galactosidase. Cells were fixed in 0.5% gluteraldehyde dissolved in PBS for 15 mins at room temperature. Next, cells were washed 1mM magnesium chloride solution in PBS. Cells were stained with β-galactosidase staining solution consisting of 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 1mg/mL X-gal in N,N dimethyl formamide (Affymetrix, 10077), in PBS with 1mM magensium chloride, pH 5.5, for 5-6 hours at 37C. Cells were then

washed with PBS, water, and 25% glycerol in water was applied prior to imaging on an Evos microscope (Life Technologies).

Cell lines used

We used the following cell lines: GL261 cells were a kind gift from Dr. David Zagzag (New York University Langone Medical Center). The following lines were purchased from ATCC: U87-MG (HTB-14), T98G (CRL-1690), A172 (CRL-1620), U138 (HTB-16). All cells were cultured in DMEM complete medium, containing 10% FBS and 1% penicillin/streptomycin. For the EPZ015666 cell line screen (below), the following additional cell lines were used: T47D cells were acquired from ATCC (ATCC HTB-133), cultured in RPMI, 10% FBS and 0.2 U/ml bovine insulin (Sigma, I6634). ZR-75-1 cells were acquired from ATCC (ATCC CRL-1500), cultured in RPMI, 10% FBS. U-2 OS cells were acquired from ATCC (ATCC HTB-96), cultured in DMEM complete medium. KELLY cells were gifts from Alexandre Puissant and Kimberly Stegmaier (Dana-Farber Cancer Institute) and were kept in DMEM complete medium MEWO cells were acquired from ATCC (ATCC HTB-65), cultured in EMEM, 10% FBS. MCF-7 cells were acquired from ATCC (ATCC HTB-22), cultured EMEM, 10% FBS and 0.01 mg/l bovine insulin (Sigma, I6634). LN229 cells were acquired from ATCC (CRL-2611), cultured in DMEM complete medium. LN-18 cells were a gift from Hiroaki Wakimoto and Daniel Cahill (Massachusetts General Hospital) and were cultured in DMEM complete. WM2664 cells were acquired from ATCC (ATCC CRL-1676), and cultured in EMEM, 10% FBS. HCT-116 cells were a kind gift from Karl Merrick (Massachusetts Institute of Technology) and cultured in DMEM complete medium SKMEL2 cells were acquired from ATCC (ATCC HTB-68) and cultured in EMEM, 10% FBS. PANC-1 cells were acquired from ATCC (ATCC CRL-1469) and cultured in DMEM complete medium. SKNAS cells were a kind gift from Alexandre Puissant and Kimberly Stegmaier (Dana-Farber Cancer Institute) and were kept in DMEM complete medium.

EPZ015666 Cell line screen

EPZ015666 was diluted in DMSO at concentrations ranging from 0-500mM (12 concentrations total) in a 96 well plate. Cells were plated (at different numbers, according to their individual proliferation kinetics) and allowed to adhere for 24 hours. Cells were then treated with 100nL of the EPZ015666 dilutions using a pin tool (V&P Scientific,

CA, mounted onto a Tecan Freedom Evo 150 MCA96 head, Tecan, CA). Following incubation for 120 hours, cell confluence in each well was estimated using the IncuCyte ZOOM microscope (Essen Bioscience).

Exploring Genetic and Epigenetic Mediators of Response to Glioblastoma Therapy

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M.S. and C.B. conducted *in vitro* and *in vivo* experiments. J.M-F. contributed to Figure 3.2. R.N. and C.C. contributed to Figure 3.4. M.S., C.B., M.H., J.L. designed the study and analyzed the data. M.S. and J.L. wrote the paper. Data associated with Figure 3.2 was published as part of [199].

■ 3.1 Abstract

THE frontline drug for glioblastoma (GBM) treatment is the chemotherapeutic agent temozolomide (TMZ). Although its addition to the treatment regimen for GBM has significantly improved survival, prognosis for patients remains extremely poor. Here, we explore the genetic and epigenetic mechanisms contributing to TMZ response in an *in vivo* GBM model using orthotopic transplantation of GL261 cells. We demonstrate that these tumors are sensitive to TMZ and irradiation therapy, alone or in combination, and that TMZ responsiveness is a function of O6-methylguanine-DNA-methyltransferase (MGMT) expression. By modulating the levels of an enzyme involved in the mismatch repair pathway, we demonstrate that small changes in expression are sufficient to drive significant resistance to TMZ *in vivo*. Furthermore, we present initial validation experiments of an *in vivo* shRNA screen identifying the epigenetic regulator PRMT5 as an important mediator of GBM response to TMZ treatment. These results demonstrate that the GL261 system can be modulated by single shRNAs, resulting in measurable effects on tumor cell composition and animal survival. Further studies will be required to understand how targeting specific epigenetic regulators identified in the screen may enhance sensitivity of tumors to TMZ treatment.

■ 3.2 Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults [144]. This devastating disease is highly aggressive and patients respond poorly to treatment. Conventional therapy involves surgical resection, followed by radiation and chemotherapy with the oral alkylating agent temozolomide (TMZ). While TMZ increases median survival from 12.1 to 14.6 months [265], benefit is invariably limited by the development of resistance.

The mechanism of action of TMZ involves methylation of DNA bases, resulting in activation of several repair mechanisms. The most toxic TMZ adduct, methylated O6-G, is normally removed by the enzyme O6-methylguanine-DNA-methyltransferase (MGMT) [321]. However, in the absence of MGMT, the persistence of the methylated base results in a mismatch during replication. This new lesion is recognized by members of the mismatch repair (MMR) pathway, which unsuccessfully attempt to repair it. The resulting process of "futile MMR" leads to clastogenic lesions upon further rounds of replication, leading to cell cycle arrest or cell death [205,238]. Consequently, mechanisms of resistance to TMZ include increased MGMT expression or mutations of the MMR components [239]. Expression of MGMT is epigenetically regulated, with approximately half of GBM patients harboring MGMT promoter methylations [132,133]. Loss of MMR proteins is also associated with tumor resistance and progression in response to TMZ therapy [38,317].

As exemplified by the regulation of MGMT expression, epigenetics can play an important role in therapy response in GBM. The complex epigenetic landscape of GBM tumors and its contribution to tumorigenesis and treatment resistance is beginning to emerge [35,41,57]. Epigenetic changes in tumors, leading to remodeling of the chromatin landscape, may be critical for determining response to conventional chemotherapeutic agents [80]. Thus far, epigenetic therapies tested in the clinic for GBM and other tumors have mostly consisted of broad inhibitors of DNA methylation and histone deacetylases [156,169]. As we gain a more precise understanding of how individual epigenetic regulators can become altered in cancer, we can begin to target them more specifically and enhance sensitivity to conventional chemotherapies [80]. This has the potential of giving rise to new combination therapies that prolong patient survival.

In vivo shRNA screens have previously been used to identify modulators of chemotherapy response [235]. Reasoning that the effect of TMZ could be modified by certain epi-

genetic regulators, we employed a loss-of-function *in vivo* shRNA screen to identify ones most relevant to GBM. We used an orthotopic transplantation model of the disease, and verified that tumor response to TMZ could be modulated by genetic manipulation. As part of a larger screen discussed in Chapter 2 of this thesis, we identified the protein arginine methyltransferase PRMT5 as being important for GBM growth in response to TMZ, both *in vitro* and *in vivo*. Our results thus far show that targeting PRMT5 enhances sensitivity to TMZ *in vitro*, but not *in vivo*.

■ 3.3 Results

■ 3.3.1 Characterizing the response of GL261 cells to therapy *in vitro* and *in vivo*

We chose the GL261 murine GBM cell line as an appropriate system for performing parallel *in vitro* and *in vivo* shRNA screens. Since the tumor microenvironment is known to be an important contributor to therapeutic efficacy [125, 237], we reasoned that a syngeneic model would be most appropriate for studying the TMZ response. As detailed in Chapter 2 of this thesis, intracranial transplantation of GL261 cells into syngeneic hosts resulted in tumors with histological characteristics of human GBM tumors. To assess the feasibility of using the GL261 transplantation model for studying mediators of sensitivity and resistance, we first tested the baseline responses of GL261 tumors to conventional therapies used in GBM. We performed intracranial transplantations of GL261 cells into syngeneic hosts, and treated the animals with TMZ as a single agent, with irradiation, or in combination. We observed that while each of the individual treatments extended survival, the combination therapy had an even stronger effect (Figure 3.1A), similar to what is observed in human patients [265]. To explore the mechanisms underlying sensitivity to TMZ, we tested GL261 cells for expression of Mgmt and found this enzyme to be absent in cell extracts (Figure 3.1B). Using *in vitro* competition assays, we asked whether restoring Mgmt protein expression would alter the sensitivity of GL261 cells to TMZ. Mixed populations of cells in which a proportion had been transduced to express an Mgmt cDNA and a GFP marker were treated with different concentrations of TMZ *in vitro* and assessed by flow cytometry 96 hours following treatment (Figure 3.1C). The proportion of GFP expressing cells could be enriched, depleted, or unchanged, indicating that the vector conferred resistance, sensitivity, or no effect to TMZ treatment, respectively. We found that Mgmt overexpression led to an
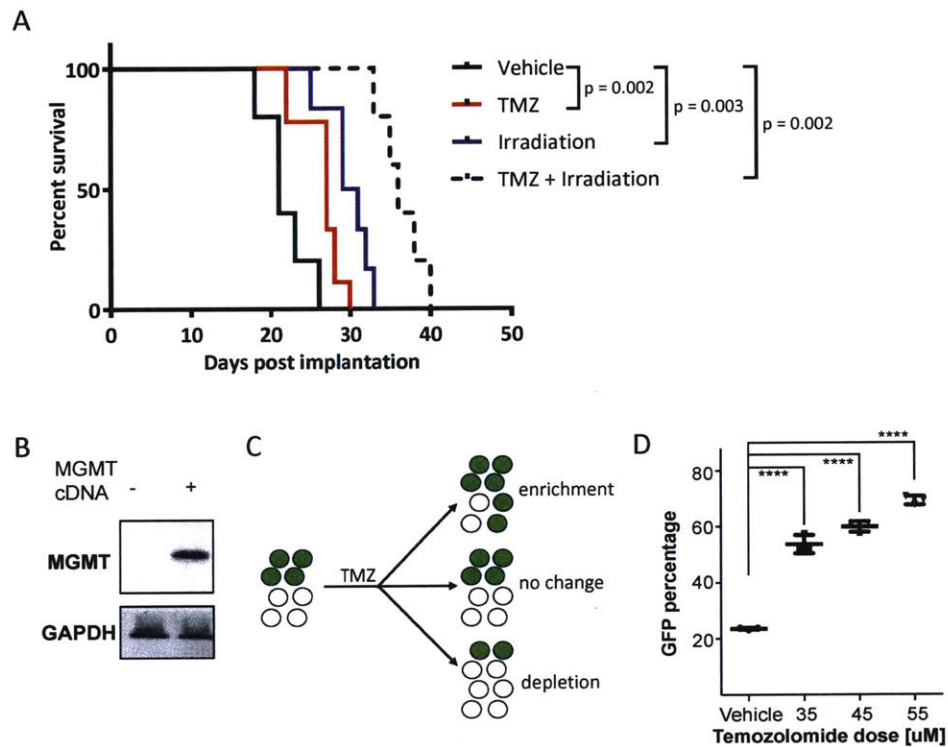
Figure 3.1. Assessing the effect of therapy on GL261 cells *in vitro* and *in vivo*. (A) GL261 cells were transplanted intracranially into syngeneic mice and treated 8 days later with vehicle (n=5), TMZ (50 mg/kg, n=9), irradiation (5 Gy, n=6), or a combination of TMZ (50mg/kg, n=5) and irradiation (5 Gy). (B) GL261 cells probed for Mgmt before and after Mgmt overexpression. (C) Scheme of *in vitro* competition assay in response to TMZ. (D) *In vitro* competition assay using mixed populations of cells, a proportion of which are transduced with an Mgmt overexpression vector that is also GFP+. Doses of TMZ represent a range of IC70-IC90, and cells were analyzed by flow cytometry after 96h.

increase in the percentage of GFP expressing cells following drug treatment, indicating that Mgmt expression could confer TMZ resistance in GL261 cells (Figure 3.1D).

■ 3.3.2 Msh2 mediates resistance to TMZ

We reasoned that competition experiments using cell based assays could be extended to tumors and used to assess modifiers of drug responsiveness *in vivo*. *In vitro* experiments demonstrated that small decreases in the levels of MSH2, an enzyme involved in the MMR pathway, could reduce MMR activity and confer TMZ resistance in human GBM cell lines [199]. We first tested the effects of Msh2 knockdown on TMZ response in GL261 cells *in vitro*. We targeted Msh2 using two constitutive shRNAs that conferred a low level of knockdown (Figure 3.2A). Competition assays were performed both *in vitro* and *in vivo* (Figure 3.2B). Following TMZ treatment *in vitro*, the proportion of shRNA expressing cells was higher in each of the two populations containing Msh2 hairpins compared to a vector control, indicating that Msh2 knockdown conferred resistance to TMZ. *In vivo*, we treated tumor bearing mice with TMZ or vehicle 8 days following tumor cell implantation. Tumors were removed when the mice were moribund and cellular composition was analyzed by flow cytometry to compare the GFP proportions in the final tumor compared to the initial mixed population (Figure 3.2C). Msh2 hairpin-expressing cells were significantly enriched in the final tumors in response to TMZ, but not vehicle (Figure 3.2D). Critically, even a low level of Msh2 knockdown resulted in a measurable effect on tumor composition *in vivo*, underscoring the importance of an intact MMR pathway for controlling the effects of TMZ in GBM tumors. Overall, these results also demonstrate that shRNAs can be used to modulate drug responsiveness in the GL261 system both *in vitro* and *in vivo* and that a single shRNA can have a measurable effect on tumor composition.

■ 3.3.3 PRMT5 loss mediates sensitivity to TMZ *in vitro* and extends survival *in vivo*

The results presented here, together with experiments outlined in Chapter 2 of this thesis, offer support for using the GL261 transplantation system for *in vivo* shRNA screening to find mediators of GBM tumor growth and TMZ responsiveness. We identified PRMT5 as being important not only for GBM tumor growth, but also as required for tumor viability specifically in response to TMZ compared to vehicle treatment. There-
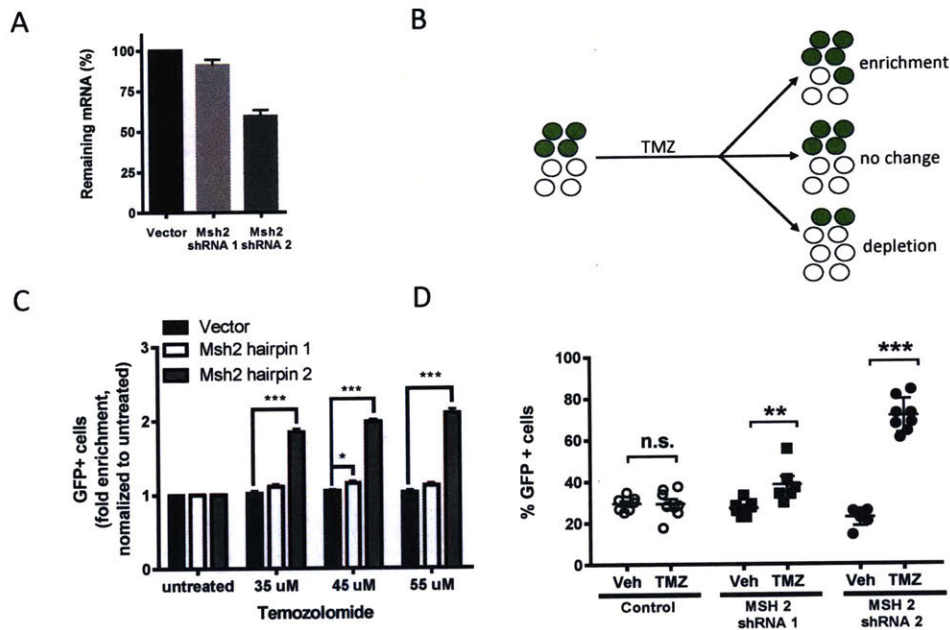
Figure 3.2. Msh2 confers resistance to GL261 cells and GL261 tumors upon TMZ treatment. (A) Msh2 mRNA levels in GL261 cells expressing a vector control or one of two Msh2 shRNAs (n=3; error bars represent SEM). (B) Scheme of *in vivo* competition assay to assess effects of Msh2 knockdown on responsiveness to TMZ in GL261 tumors. GL261 cells express GFP as a marker for shRNA expression. (C) *In vitro* competition assay demonstrating increases in the proportion of Msh2 shRNA, GFP-expressing cells in response to TMZ treatment at three different doses, compared to a vector control. Dose of TMZ represent a range of IC70-IC90, and cells were analyzed by flow cytometry after 96h (n=3; SEM; * P < 0.05; *** P < 0.001 Student t test). (D) Results of *in vivo* competition assay, showing enrichment of Msh2 shRNA, GFP-expressing cells in response to TMZ treatment in GL261 tumors. Mice were treated with 50mg/kg TMZ 8 days after tumor cell implantation (SD; * P < 0.05; *** P < 0.001 MannWhitney test).
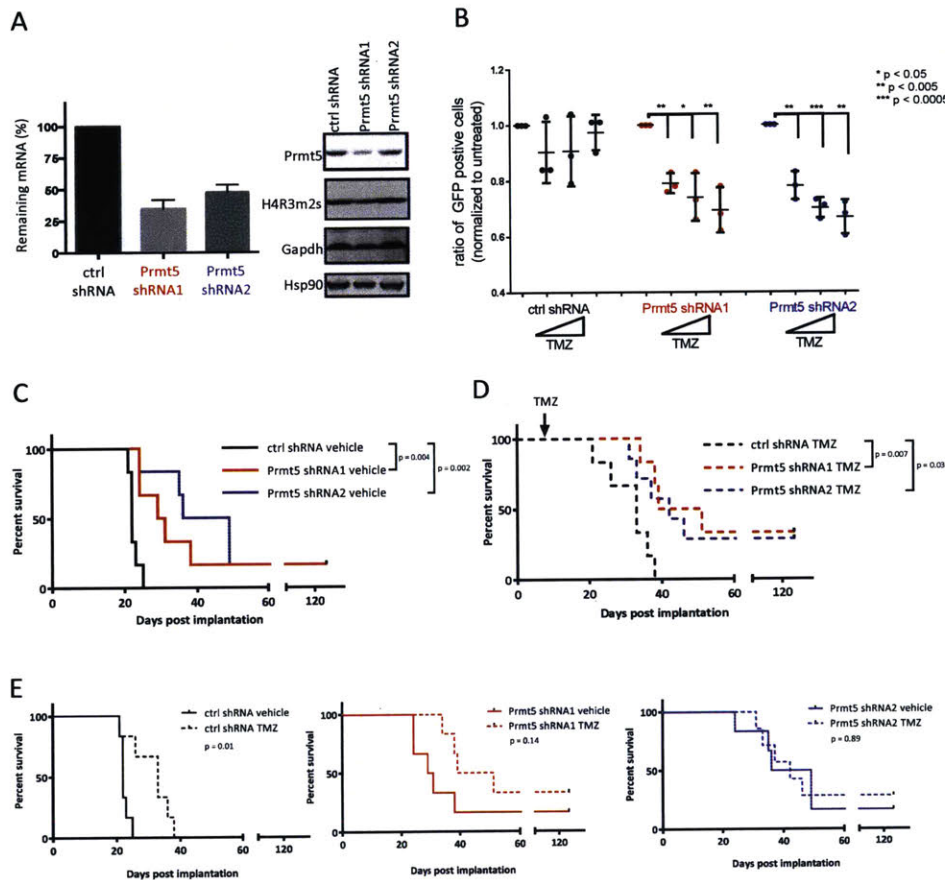
Figure 3.3. Assessing a role for PRMT5 in mediating sensitivity to TMZ *in vitro* and *in vivo*. (A) Prmt5 mRNA and protein levels (left and right, respectively), in GL261 cells expressing a constitutive Prmt5 or control shRNA (n=3; error bars represent SEM). H4R3me2s = symmetric dimethylarginine H4R3. (B) *In vitro* competition assay demonstrating sensitivity to TMZ in Prmt5 shRNA expressing cells, compared to those expressing a control shRNA. TMZ concentrations used: $0\mu M$, $20\mu M$, $35\mu M$, $50\mu M$, cells were analyzed by flow cytometry after 6 days. (C) to (E) Survival plots of one *in vivo* experiment consisting of 6 cohorts of mice, separated into different plots for clarity. Mice were implanted intracranially with GL261 cells expressing a constitutive Prmt5 shRNA or a control shRNA. (C) Animals were treated with vehicle or (D) TMZ (50mg/kg) at day 9 post tumor cell implantation. (E) Pairwise comparisons between vehicle and TMZ-treated groups of mice bearing GL261 tumors containing the same shRNA. P value determined by Log-Rank (Mantel-Cox) test, and n=6 for all groups except for Prmt5 shRNA2 TMZ, for which n=7.

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fore, we sought to validate these results and first tested whether genetic depletion of PRMT5 could sensitize GBM cells to TMZ *in vitro* and *in vivo*. Knockdown of Prmt5 expression using two different constitutive shRNAs in GL261 cells led to a reduction in mRNA and protein levels. It also led to decreased levels of symmetrically dimethylated H4R3, a marker of Prmt5 methyltransferase activity (Figure 3.3A). Using *in vitro* competition assays, Prmt5 knockdown was found to enhance sensitivity to TMZ, in a concentration-dependent manner (Figure 3.3B). Next, pure populations of GL261 cells expressing PRMT5 shRNAs or a control shRNA were transplanted intracranially into syngeneic mice, and treated with TMZ or vehicle. Knockdown of Prmt5 alone resulted in a significant extension of lifespan (Figure 3.3C). TMZ treatment of Prmt5 depleted tumors showed a survival benefit compared to treatment of tumors harboring normal levels of Prmt5 (Figure 3.3D). This effect appeared to be driven by Prmt5 loss, as there were no significant survival differences between Prmt5 knockdown tumors treated with vehicle versus TMZ (Figure 3.3E). These data validate that Prmt5 is indeed important for GBM tumor growth, suggesting its potential as a therapeutic target. Furthermore, these results demonstrate that mice bearing tumors with low Prmt5 expression levels survive longer, irrespective of TMZ treatment, compared to mice bearing control tumors that are treated with TMZ. Taken together, these data show that loss of Prmt5 expression sensitizes GL261 cells to TMZ treatment *in vitro*, but not *in vivo*. Even though these results do not demonstrate a benefit from targeting tumors with TMZ and Prmt5 knockdown, they highlight PRMT5 as an appealing therapeutic target.

■ 3.3.4 EPZ015666 treatment of GL261 GBM tumors leads to loss of methyltransferase activity without affecting survival

The recent identification of a highly specific and orally available small molecule inhibitor for PRMT5, EPZ015666 [44], offered the unique opportunity for testing combination therapy with TMZ *in vivo*. We sought to assess whether PRMT5 could be targeted in GBM tumors by EPZ015666 and whether TMZ co-treatment with this drug would affect survival. We first tested whether EPZ015666 was capable of inhibiting PRMT5 activity *in vivo*. As the blood brain barrier (BBB) permeability of this drug was unknown, we treated GL261 tumor-bearing mice for short periods of time prior to euthanasia due to high tumor burden, reasoning that the BBB was most likely to be disrupted at this time. Indeed, as assessed by immunoblotting for symmetric dimethylarginine (SDMA) marks, we observed that EPZ015666 effectively inhibited PRMT5 methyltransferase
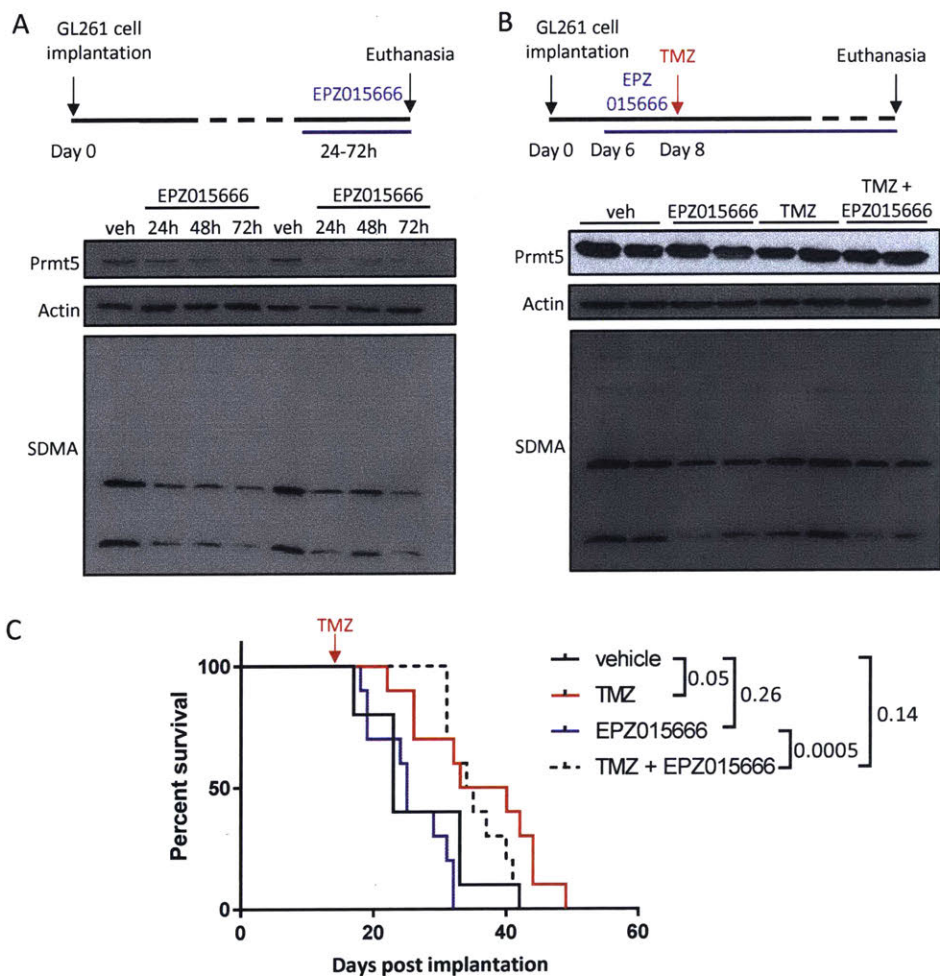
Figure 3.4. Treatment of GL261 tumor-bearing mice with PRMT5 inhibitor results in loss of methyltransferase activity, but not increased survival. (A) Mice were intracranially transplanted with GL261 cells, and treated with vehicle or PRMT5 inhibitor EPZ015666 for 24h, 48h, or 72h before euthanasia due to high tumor burden. The extracted tumors were assessed for symmetric dimethyl arginine (SDMA) as an indicator of inhibition of PRMT5 methyltransferase activity. Each lane represents a tumor from a different animal. (B) In a separate experiment mice were intracranially transplanted with GL261 cells, and divided into 4 groups, to be treated with vehicle, TMZ, EPZ015666, or TMZ and EPZ015666. TMZ treatment was administered as a one time ip injection at 50mg/kg at day 8, and EPZ015666 was administered 2x/day orally at 100mg/kg, starting on day 6 and continuing until the mice were euthanized due to high tumor burden. Two tumors from each cohort were extracted and assessed for inhibition of PRMT5 methyltransferase activity by immunoblot. (C) Survival analysis, n=10 for each group. P value determined by Log-Rank (Mantel-Cox) test.

activity *in vivo* (Figure 3.4A). Next, we transplanted mice intracranially with GL261 cells and divided them into four groups, to be treated with vehicle, TMZ, EPZ015666, or TMZ combined with EPZ015666. We began treating animals with the PRMT5 inhibitor starting on day 6 after cell implantation to allow time for tumor cell engraftment (Figure 3.4B). In order to maximize the exposure of the tumor to the drug, we treated animals until they required euthanasia due to high tumor burden. TMZ treatment was performed as a one-time injection at 8 days post tumor cell implantation. By extracting tumors from moribund mice, we confirmed that EPZ015666 treatment either alone or in combination with TMZ resulted in decreased levels of SDMA (Figure 3.4B). Using this treatment regimen, we did not observe any survival benefit from treatment with EPZ015666 alone, or the combination therapy compared to vehicle-treated controls (Figure 3.4C). Addition of TMZ to EPZ015666 treatment improved survival compared to EPZ015666 alone. Although these results require further validation, the experiments presented here demonstrate that EPZ015666 is capable of inhibiting PRMT5 methyltransferase activity in GBM tumors *in vivo* with rapid kinetics.

■ 3.4 Discussion

The addition of TMZ to the treatment regimen of GBM patients over a decade ago offered major improvements for the treatment of this aggressive disease [265]. TMZ can be administered orally and has a relatively mild side effect profile [291]. However, as resistance invariably arises in GBM tumors, identifying epigenetic mediators of this process could lead to combination therapies for improving the efficacy of TMZ.

We first tested the response of the GL261-derived intracranial tumors to TMZ and found that, similar to human GBM tumors, they were sensitive to TMZ and irradiation. Survival was even further extended by a combination treatment. We showed that drug sensitivity resulted from absence of MGMT expression in GL261 cells, as its restoration drove resistance to TMZ *in vitro*. We further demonstrated that TMZ resistance could be modulated *in vivo* by the expression of a single shRNA, by targeting the MMR enzyme Msh2. Importantly, these findings also suggest that even small decreases in a component of the MMR pathway can drive tumor resistance to TMZ, an observation that has therapeutic implications for GBM patients [199].

From parallel *in vitro* and *in vivo* shRNA screens for modulators of TMZ responsiveness, we identified PRMT5 as being important for driving resistance to TMZ. In

validation experiments *in vitro*, stable PRMT5 knockdown did indeed increase sensitivity of GL261 cells to TMZ. Only one other report has previously tested the effects of PRMT5 modulation on TMZ response, showing that pretreatment of GBM cells with TMZ increased responsiveness to PRMT5 siRNA-mediated knockdown [312]. Our results demonstrated that *in vivo*, PRMT5 knockdown was sufficient to significantly extend lifespan, although treatment with TMZ did not confer an added benefit. Targeting PRMT5 activity in tumors using a small molecule inhibitor did not enhance survival as a monotherapy, and it did not increase the effectiveness of TMZ.

It is important to understand why the results presented here do not support the original identification of PRMT5 as a possible mediator of responsiveness to TMZ. One of the greatest challenges to *in vivo* screening pertains to the extrapolation of screening results to validation experiments [33]. Specifically, cells containing an shRNA targeting a specific gene may act differently in the context of a mixed population, such as the one in the original screen, compared to pure populations of single shRNAs, as is the case in validation experiments. The presence of long term survivors in tumors arising from constitutive PRMT5 knockdown cells, irrespective of TMZ treatment, may indicate that PRMT5 is important for tumor cell engraftment. This notion is consistent with PRMT5 arising as a top hit in the screen, even in the absence of TMZ. The fact that PRMT5 also appears to be specifically important in TMZ-treated tumors may further support the requirement for PRMT5 as being required for tumor cell engraftment. If the tumors cells, once originally engrafted after implantation, were then subjected to another bottleneck owing to TMZ treatment, cells containing PRMT5 shRNAs would be selected against on two separate occasions. Their specific depletion would be more apparent in the context of a mixed cell population in the original screen, as opposed to the validation experiments. To test this hypothesis, it would be important to check the levels of PRMT5 and its associated methyl marks in the final tumors in the single shRNA experiments. It is possible that cells containing lower levels of PRMT5 knockdown, and therefore higher residual PRMT5 expression, would be positively selected following TMZ treatment and repopulate the tumor.

In GL261 tumors, the use of a PRMT5 inhibitor *in vivo* did not affect survival, irrespective of TMZ treatment. It should be noted, however, that although we verified that EPZ015666 is capable of inhibiting PRMT5 activity in GBM tumors, we do not yet know whether it is able to cross the BBB, limiting the interpretation of our results. It is possible that despite long term treatment, if the drug is only able to access the

tumors when the BBB is sufficiently disrupted, this may not allow sufficient time for it to have an effect on survival. Although in this cohort of animals TMZ did improve survival, the effect was not as pronounced as in other experiments, indicating a possible technical limitation in this particular study. Therefore, in isolation, these data do not allow us to conclude why treatment with EPZ015666 was ineffective in the GL261 GBM tumors.

Overall, further studies will be required to understand the role that epigenetic regulators can play in mediating sensitivity and resistance to conventional chemotherapies in GBM. Understanding these interactions and their molecular underpinnings will have important implications for combination therapies for a disease whose prognosis remains abysmal.

■ 3.5 Materials and Methods

Reagents

Temozolomide (TMZ) was purchased from Sigma (T2577) and dissolved in DMSO. For *in vivo* experiments, TMZ was further diluted in sterile 0.9% sodium chloride solution. EPZ015666 was purchased from DC Chemicals (DC8012) and resuspended in DMSO for *in vitro* experiments. For *in vivo* experiments, EPZ015666 was dissolved in 0.5% methylcellulose in water.

Competition Assays

GL261 cells were a kind gift from Dr. David Zagzag (New York University Langone Medical Center). Cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin/streptomycin. GL261 cells were infected *in vitro* with the indicated vectors and sorted by fluorescence activated cell sorting (FACS) to generate pure populations of transduced cells. These populations were then mixed with untransduced cells such that 20-40% of the cells express the vector. For *in vitro* competition assays, cells were seeded and treated with TMZ 24 hours later. After 4 days or 6 days (as indicated), the proportion of GFP expressing cells was assessed by flow cytometry. For *in vivo* competition assays, a mixed population of 500,000 GL261 cells, same as ones used for *in vitro* assays, were transplanted intracranially into C57BL/6 mice. Animals were treated with TMZ or vehicle at 8 days post tumor cell transplantation (50mg/mg). When the mice showed signs of high tumor burden according to predefined criteria, they were euthanized and the brains were extracted. The tumors were excised and dissociated into a single cells suspension using a Brain Tumor Dissociation Kit from Miltenyi Biotech (130-095-942) and using a gentleMACS Dissociator (Miltenyi Biotech) according to manufacturer recommendations. The cells were then analyzed by flow cytometry to determine the percentage of GFP expressing cells using a BD LSR II machine.

Animal studies

C57BL/6 female mice aged 6-8 weeks were purchased from Jackson Laboratories (Bar Harbor, MA). GL261 cells were harvested by trypsinization, resuspended and counted.

Intracranial transplantations were performed by injecting 500,000 cells in sterile PBS into the left hemisphere, approximately 2mm to the left of the bregma along the coronal suture. Mice were treated with TMZ administered by intraperitoneal injection at a dose of 50mg/kg of body weight at 8 days post tumor cell implantation. EPZ015666 was administered by oral gavage every 12 hours at a dose of 100mg/kg of body weight for the indicated periods. When irradiation was performed, mice were irradiated with a dose of 5Gy at 8 days post tumor cell implantation. All animal studies were performed in accordance with MIT Committee on Animal Care protocol approval.

Vector constructs and shRNAs

Mouse MGMT cDNA (Origene, MC202089) was cloned into the retroviral vector pMIG using the following primers:
forward primer: 5'-GCGATCTCGAGACGCATGGCTGAGACCTGCAAAATGA-3'
reverse primer: 5'-GCATCGAATTCCAGATCAATTTCGGCCAGACGGCTCA-3'
pMIG was a kind gift from William Hahn (Addgene plasmid 9044). For shRNA experiments, the following shRNAs targeting mouse Msh2 were cloned into the TMP retroviral vector, as described previously (Dickins et al., 2005). An empty TMP vector was used as a control.
MSH2 shRNA 1: 5'-CAGGATGCCATTGTTAAAGAA
MSH2 shRNA 2: 5'-AACGATGTGCTGGCTCACTTA
The following shRNAs targeting mouse Prmt5 or Renilla luciferase as a control were cloned into the the constitutive retroviral vector MSCV/LTR/miR30/SV40/GFP (MLS).
Prmt5 shRNA1 (PRMT5.2291) - 5'-GCCCGGTCTACCTAGCAAGTTT
Prmt5 shRNA2 (PRMT5.1153) - 5'-GACCGAGTACCAGAAGAAGAAA
Ctrl shRNA (Ren.713) - 5'-TAGGAATTATAATGCTTATCTA

Immunoblotting

For *in vitro* studies, cells were harvested by scraping on ice and lysed with RIPA buffer. For *in vivo* studies, tumors were isolated from mice upon euthanasia due to high tumor burden or at the indicated timepoint. Brains were extracted and tumors isolated from the surrounding normal tissue and snap frozen in liquid nitrogen and stored at -80C. For protein extraction, tumors were mechanically dissociated in RIPA lysis buffer using a Geno/Grinder 2010 (SPEX SamplePrep). The following antibodies

were used: MGMT (R&D, MAB3299), PRMT5 (Cell Signaling, 2252S), GAPDH (Santa Cruz, sc365062), actin (Santa Cruz, sc1616), symmetric dimethylarginine SDMA (Cell signaling, 13222). Immunoblots were detected using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) and an ImageQuant LAS4000 imager (GE) or by autoradiography film.

Chapter 4

Discussion

THE multifaceted roles of epigenetic regulators and their contribution to glioblas-
toma (GBM) are only beginning to be uncovered. In this chapter, I discuss the
results of an *in vivo* shRNA screen and the major findings demonstrating a key role for
the epigenetic regulator PRMT5 in GBM. I further consider the feasibility of PRMT5
as a therapeutic target for this disease. Finally, I examine the roles for PRMT5 in regu-
lating gene expression through splicing and reflect on the implications in the context of
existing literature. I end by considering the significance of these results and discussing
how they could be further examined in future investigations.

■ 4.1 *In vivo* RNAi screening as a method for identifying novel epigenetic mediators of GBM growth

While the utility of *in vivo* screening for the discovery of new biological pathways
and therapeutic targets has been shown in numerous different tumor models [22, 36,
201, 320, 325], such experimental approaches in GBM have only recently been pre-
sented [102, 241]. This may be due to the technical difficulties associated with achieving
consistent intracranial transplantations, as well as the requirement that a large enough
proportion of transplanted cells engraft and contribute to tumor formation. The latter
requirement is difficult to achieve within the confines of the brain, where a physical
constraint is placed on the number of cells that can be transplanted without significant
disruption of the brain parenchyma. We have addressed some of these limitations by
establishing a new model for loss of function *in vivo* RNAi screening, using orthotopic
transplantation of GL261 murine GBM cells. This system was chosen because the tu-
mor microenvironment is intact during the process of tumorigenesis, and due to its
resemblance to the human disease in terms of histology and treatment response. We

performed several experiments prior to initiating the screen to assess and establish the reliability of this model for *in vivo* shRNA screening. These consisted of limiting dilution assays to inform the size of pooled shRNA library that can be screened, as well as single shRNA experiments in the presence and absence of the chemotherapeutic agent temozolomide (TMZ) to demonstrate the dynamic range of the system. The screen produced high quality sequencing data, and from the list of candidates resulting from the screen, we identified PRMT5 as the target for further validation for three reasons. First, it was consistently identified as a top hit in both *in vitro* and *in vivo* samples, independent of TMZ treatment. This would allow for *in vitro* validation prior to moving on to animal experiments. Second, the role of PRMT5 in cancer was becoming increasingly appreciated in the literature and there were two reports demonstrating PRMT5 overexpression in GBM patients and its requirement for tumorigenesis in one xenograft model [123, 312]. Therefore, there was reason to believe that this hit could be validated and would contribute to a developing field with high therapeutic relevance for patients. Finally, we wanted to use PRMT5 as the ultimate proof of principle to demonstrate that screening for epigenetic regulators using this system and methodology could produce reliable and physiologically relevant results, opening the door for future validation of other targets. Indeed, the results presented here indicate that loss of function RNAi screens using the GL261 orthotopic transplantation model can be used to uncover novel epigenetic regulators that are highly relevant for human disease. In the future, it will be interesting to also validate other candidates from this screen.

■ 4.2 Assessing the role for PRMT5 in GBM tumors

The results of the *in vivo* studies presented here lend support to the important roles for PRMT5 in GBM and demonstrate, to our knowledge, the first use of a highly specific PRMT5 inhibitor, EPZ015666, for the treatment of a GBM model. To briefly summarize these results, we used intracranial transplantation of human and mouse GBM cells, as well as subcutaneous xenografts, to test the effects of PRMT5 knockdown or inhibition with EPZ015666 on tumor growth. We also tested whether PRMT5 loss could sensitize GBM tumors to TMZ. In Chapter 2, we describe that inducing PRMT5 shRNA expression in established U-87 MG tumors results in a selective depletion of the knockdown cells, indicating that PRMT5 loss is detrimental to tumor growth. In U-87 MG subcutaneous xenografts, PRMT5 inhibition with EPZ015666 resulted in smaller tumors, and increased survival of animals bearing intracranial xenografts. In Chapter 3,

we show that in a syngeneic model, PRMT5 constitutive knockdown extends survival of GL261 tumor-bearing animals. Treatment with TMZ did not further impact survival. In this same tumor model, treatment with EPZ015666 did not improve survival or appear to have any benefit aside from that conferred by TMZ alone. In this section, I synthesize the meaning of these results and consider the implications and caveats of these experiments. Many of these interpretations are dependent on addressing the ability of EPZ015666 to cross the blood brain barrier, and I explain our efforts to address this question at the end of the section.

■ 4.2.1 Assessing the role for PRMT5 at different stages of tumor growth

Our results support a role for PRMT5 in tumor cell engraftment and early tumor growth. In the syngeneic model where GL261 cells containing a constitutive PRMT5 shRNA are transplanted intracranially into mice, PRMT5 knockdown showed significant lifespan extension with both shRNAs, compared to a control shRNA (Figure 3.3C). These results are in line with previously reported xenograft experiments demonstrating that constitutive PRMT5 knockdown in human GBM cells can extend survival [13,312]. Interestingly, we observed long term survival of animals from both of the PRMT5 knockdown groups, as well as the two other groups where PRMT5 loss was coupled with TMZ treatment. Based on our experience with this model system, we found these long term survivors to be highly unusual, as we always observe high penetrance of tumor development in this model. This led us to hypothesize that constitutive PRMT5 knockdown affected the efficiency of tumor cell engraftment and early tumor growth. Of course, it is also possible that continued loss of PRMT5 expression additionally affects tumor progression, as discussed below.

The results of our subcutaneous U-87 MG xenografts treated with EPZ015666 (Figure 2.8C) support a role for PRMT5 in the early stages of tumor growth, as a difference in tumor size can already be appreciated after the first of two separate rounds of treatment. After the second round, this difference becomes enhanced. There are two possible explanations for this observation. One option is that the second round of treatment augments the effect of the first by independently impeding later stages of tumor growth. The results of the U-87 MG intracranial xenograft with inducible PRMT5 shRNA knockdown support this possibility (Figure 2.8A), demonstrating that PRMT5 is required for cellular fitness in tumors following engraftment. Another possibility is that the second treatment offers no additional benefit and the survival improvement

is due to senescence (as indicated by *in vitro* results, Figure 2.7D), resulting from the first round, which becomes more pronounced in comparison to the proliferating cells of the untreated tumors. Therefore, from this data, we cannot make definitive conclusions on the effect of PRMT5 inhibitor on late stages of tumor growth. A better understanding of whether the PRMT5 inhibitor is capable of crossing the intact BBB at early timepoints will help distinguish between these two possibilities. Meanwhile, the intracranial xenograft experiment showed that PRMT5 inhibitor treatment in two one week courses, once early in tumor formation, and once during later tumor growth, results in a significant lifespan extension (Figure 2.8D). Interpretation of these results is complicated by our as of yet incomplete understanding of the ability of EPZ015666 to move across the BBB. If the drug is not capable of crossing the intact BBB, it means that the intracranial tumors were only exposed to it at late timepoints, when we have evidence that the BBB is disrupted. Therefore, the observed lifespan extension would be due to the effect of the drug during this later stage and we could make the preliminary conclusion that maintenance of PRMT5 expression is important for late stage tumors. On the other hand, if the drug is capable of crossing the intact BBB, it is likely to have an effect on early tumors, but we would not be able to conclude as to any effects on maintenance, similar to the subcutaneous model. It is important to note that we started treatment of the mice on day 4 post implantation, when we believe the cells have already seeded into their transplanted location. Altogether, the *in vivo* studies of genetic and pharmacological-mediated loss of PRMT5 function point to a role for PRMT5 in GBM tumor engraftment and early tumor growth. A more definitive conclusion about the effects of PRMT5 during later stages of GBM will be dependent on a clearer understanding of the BBB permeability of EPZ015666.

■ 4.2.2 Explaining discrepancy between knockdown and inhibition of PRMT5 in the GL261 model

In the syngeneic model GBM model, constitutive PRMT5 knockdown extended animal survival (Figure 3.3C). This effect was not observed with EPZ015666 treatment administered from day 6 until euthanasia (Figure 3.4C). This discrepancy can have several possible explanations. One option is that PRMT5 is important only during tumor cell implantation and early tumor growth, as explained above. Another possibility relates to the BBB permeability of EPZ015666. From the short term *in vivo* drug treatment, we know that the drug diminishes symmetric arginine dimethylation as early as 24h *in*

vivo (Figure 3.4A). However, these experiments were performed in large tumors which most likely had a highly disrupted BBB. Therefore, in the long term treatment survival study, we cannot know when the drug began to access the tumors. If EPZ015666 is capable of penetrating the intact BBB, then the likely explanation is that PRMT5 affects tumor cell engraftment and starting treatment after this stage does not impact survival. On the other hand, if the drug does not cross the BBB and can only access tumors in the latest stages, it may not have enough time to affect survival. Another possible explanation for this outcome, related to the specific effects of PRMT5 inhibition in the context of an immunocompetent model is discussed later in this chapter. It is unlikely that the long term treatment with EPZ015666 was toxic to the animals and abrogated any beneficial effects of PRMT5 inhibition. We performed toxicological analyses on non-tumor-bearing animals, and did not note any adverse effects following 1 week of treatment (Figure 2.8B, and data not shown). Longer treatment periods, ranging from 18-21 days, were reported by Chan-Penebre and colleagues, without noting any adverse effects [44]. Overall, further investigation is warranted to determine the period during which PRMT5 knockdown or inhibition is the most efficacious. Aside from a clearer understanding of the BBB permeability of EPZ015666, one way to address this would be to transplant GL261 cells with an inducible PRMT5 shRNA and bypass the effects of PRMT5 on tumor cell engraftment that we observed with a constitutive knockdown. We previously attempted this experiment, but experienced technical limitations due to a leaky vector that activated PRMT5 knockdown even in the absence of doxycycline treatment. This experiment can be modified with the highly reliable TRMPVIR doxycycline inducible system successfully used for the Kras shRNA experiments (Figure 2.1).

■ 4.2.3 Addressing the BBB permeability of EPZ015666

Many of the above interpretations depend on addressing whether EPZ015666 is capable of crossing the intact BBB. We previously attempted to address this question by performing intracranial transplantations of U-87 MG cells, and euthanizing mice at different time points thereafter. Groups of mice were treated with either Prmt5 inhibitor or vehicle for 3 days before euthanasia, a time window known to be sufficient for depleting PRMT5-dependent symmetric dimethylation in response to the drug *in vivo*. We performed extensive histological characterization using a marker for PRMT5 activity, H4R3me2s. We also coupled this analysis with assays to determine BBB integrity at these same timepoints of tumor development. This analysis showed that EPZ015666

treatment results in decreased H4R3me2s staining in large tumors. However, due to extensive variability of staining, we were unable to firmly conclude whether the drug crosses the BBB to affect the histone mark in smaller tumors. In order to better assess drug distribution in the tumor and normal brain, we are collaborating with Dr. Nathalie Agar's group to perform matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) [188, 233]. Understanding the ability of the drug to cross the BBB has important implications for human clinical treatment, since the highly infiltrative nature of GBM cells means that many cells outside the main tumor mass are surrounded by largely normal brain parenchyma [233].

■ 4.3 Interpreting gene expression signatures following PRMT5 inhibition

Extensive functional pathway analysis of the gene expression changes following PRMT5 inhibitor treatment in U-87 MG cells was undertaken using different tools and showed highly concordant results. These analyses revealed several important signatures, some of which agree and others which conflict with previously described roles for PRMT5. Most strikingly, these data showed increased reorganization of the extracellular matrix and signatures consistent with the epithelial to mesenchymal transition (EMT), p53 signaling, inflammatory responses and NF-κB signaling, as well as decreased cell cycle progression.

In vitro analyses as well as the RNAseq results showed decreased proliferation and a block in cell cycle progression in response to EPZ015666, consistent with widely described roles for PRMT5 in GBM and other tumors [44, 182, 312]. Although we observed an increase in p53 signatures, as has been previously reported upon PRMT5 deletion [23, 166], we did not find increased apoptosis upon PRMT5 inhibition *in vitro*, inferred by looking at the subG1 fraction. However, this is not the most sensitive method and may not accurately detect apoptotic cells following G2/M arrest. A more thorough analysis could be undertaken by performing flow cytometry using Annexin V, as well as immunoblotting for markers of apoptosis, such as caspases and pro-apoptotic Bcl-2 family members. Interestingly, we identified increased senescence in response to drug treatment, consistent with a recent study reporting senescence as a consequence of PRMT5 knockdown in human glioma neurospheres, but not adherent GBM cells [13]. The authors find that PTEN is repressed by PRMT5, and that in response to PRMT5 loss, PTEN facilitates senescence through AKT and p27. However, U-87 MG cells are

mutant for PTEN [56], suggesting a PTEN-independent mechanism driving senescence, perhaps through mTOR-mediated upregulation of p53 [13, 212].

It is important to note that our results indicating that PRMT5 inhibition in GBM cells leads to NF-κB pathway activation are in conflict with several reports demonstrating that PRMT5 directly methylates the p65 subunit of NF-κB, leading to its transcriptional activity [127, 128, 302]. However, another report shows that PRMT5 is required for the induction of TRAIL-dependent but not TNF-α-dependent NF-κB signaling [274]. Two studies by Harris and colleagues also reported decreased expression of inflammatory chemokines in endothelial cells in response to PRMT5 depletion [127, 128], in stark contrast to results reported in GBM [312], as well as our findings. Therefore, PRMT5 inhibition may impact NF-κB signaling in a tissue or context-specific manner.

■ 4.3.1 PRMT5 and EMT

A mass spectrometry experiment for PRMT5 interacting proteins in T cells revealed that a large number of proteins involved in cytoskeletal rearrangement are symmetrically dimethylated [288]. Our observations that PRMT5 modulates the emergence of mesenchymal cellular characteristics echo those described in lung and breast cancer cell lines upon PRMT5 or MEP50 knockdown by Chen and colleagues [49]. The authors explored the role of PRMT5 in EMT, and through functional pathway analyses showed that knockdown of PRMT5 or MEP50 led to an upregulation of extracellular matrix organization and cell adhesion pathways, as well as a set of predicted upstream regulators that is highly overlapping with our own. Where our results diverge from theirs is in the description of the consequences of these transcriptional changes. The Chen study described that PRMT5 and MEP50 were necessary for mediating TGF-β dependent migration and invasiveness in lung and breast cancer cell lines. In response to TGF-β, wild type cell lines increased migration and showed markers consistent with EMT (loss of CDH1, gain in VIM and SNAIL). Meanwhile, PRMT5 knockdown led to reduced proliferation, as well as decreased cell migration and invasion, and notably, diminished levels of SNAIL. Treatment of PRMT5/MEP50 knockdown cells with TGF-β did not rescue the migration and invasion defects, nor did it lead to the expression of EMT markers. From these results, the authors conclude that the expression of the TGF-β induced EMT phenotype is dependent on PRMT5/MEP50 [49]. Meanwhile, although our data also show that PRMT5 knockdown and inhibition results in decreased proliferation in GBM cells, our transcription analysis seems to indicate a gain of mesenchymal char-

acteristics, including SNAIL, upon PRMT5 inhibition. PRMT5 was previously shown to associate with AJUBA and SNAIL to form a repressive complex that downregulates E-cadherin expression [137]. While this study notes an upregulation of E-cadherin protein following PRMT5 knockdown, they do not report the effects on expression of SNAIL. Gene expression analysis of 40 NSCLC cell lines identified PRMT5 as being highly expressed in cell lines showing features of EMT (VIM high, CDH1 low), and patient samples with higher PRMT5 protein levels exhibited loss of epithelial markers and a poor prognosis [147]. While these studies point to a role for PRMT5 as a positive regulator of EMT, it is possible that the effects of PRMT5 in EMT induced by TGF-β in epithelial tumors are different from those leading to an increased mesenchymal subtype in GBM cells in response to NF-κB signaling. PRMT5 may be involved in yet unidentified transcriptional complexes in a cell type specific manner. Therefore, the link between NF-κB signaling and EMT in response to PRMT5 inhibition in our system remains to be clarified.

■ 4.3.2 NF-κB pathway activation and EMT signatures point to acquisition of a mesenchymal phenotype

Subclassification of GBM tumors based on genetic and epigenetic profiles has revealed that this disease is composed of different subtypes with distinct molecular characteristics, therapeutic vulnerabilities and clinical prognoses [41, 232, 290]. Therefore, our observations that a shift in clinical subtype occurs upon PRMT5 inhibitor treatment are important not only for dissecting the molecular pathways affected by PRMT5 in GBM, but also for revealing further therapeutic options and likely outcomes.

Several of the pathway analyses related to our gene expression results show that PRMT5 inhibition by EPZ015666 results in increased NF-κB signaling, extracellular matrix remodeling, and, possibly, EMT. While PRMT5 has previously been implicated to have a role in TGF-β-induced EMT in other cancer types, the gene expression data seems to suggest that NF-κB signaling, whose role in facilitating EMT is well appreciated [130, 143, 176] is the likely inducer of the mesenchymal phenotype in our system.

Bhat and colleagues demonstrate that the NF-κB pathway is associated with the mesenchymal GBM subtype [24], and human tumors belonging to this subclass show mesenchymal and microglial features [24, 232, 290]. Several independent pathway anal-

yses point to increased NF-κB signaling in our dataset upon EPZ015666 treatment, and we observe an enrichment for the Phillips, Verhaak, and Bhat mesenchymal signatures [24, 232, 290]. Bhat et al. note that although some of the master transcription factors in the mesenchymal signature overlap with ones known to be involved in EMT, there was no increase in the classical EMT factors SNAIL, SLUG, or TWIST [24]. While this remains to be verified by qPCR in our system, in our RNAseq dataset, we see no differential expression of SLUG, ZEB1, ZEB2, TWIST, VCAN (versican), CDH1, CDH2, or VIM (vimentin) [113], and only SNAIL is significantly upregulated in response to EPZ015666. This supports the possibility that the Hallmark EMT signature in our GSEA analysis is mostly driven NF-κB signaling as opposed to TGF-β signaling. Indeed, the Hallmark TGF-β signature was not significant in our GSEA analysis. Interestingly, SNAIL is known to be stabilized by TNF-α induced NF-κB signaling, leading to its nuclear localization and induction of EMT [299, 309]. SNAIL is a master regulator of breast cancer stem cells and EMT [315], although the roles of SNAIL in GBM are not yet not well understood. SNAIL has been described as a critical regulator of a glial to mesenchymal transition in response to irradiation [197], and its overexpression promotes invasiveness of GBM cells, while decreasing their tumorigenic potential and prolonging survival in immunocompromised mice [246]. The authors note that this decreased tumorigenicity was surprising because of the strong link between EMT and stemness [315], and they outline that one possibility is that SNAIL decouples the invasion and tumorigenic potential of GBM cells. Therefore, our data suggest that following PRMT5 inhibition, NF-κB facilitates the appearance of highly overlapping EMT and mesenchymal GBM features, with SNAIL possibly playing a role in this phenotype.

■ 4.4 Perspectives on using PRMT5 as a therapeutic target in GBM

Together, the *in vitro* analyses of PRMT5 knockdown and inhibition joint with transcriptional profiling following PRMT5 inhibition indicate decreased proliferation and progression through the cell cycle, together with the induction of senescence and features consistent with a shift towards a mesenchymal phenotype. These characteristics, reminiscent of EMT, appear to be correlated with NF-κB signaling and an increased immune signature. It is possible that increased SNAIL expression leads to a decoupling of invasiveness from proliferation and tumorigenic potential. This may also offer an alternative explanation for the observed discrepancy between genetic loss and pharma-

cological inhibition of PRMT5 *in vivo*.

Since EPZ015666 has never been studied in GBM, and previous studies of PRMT5 knockdown in GBM were performed in xenograft models [13, 312], it is important to entertain all possible explanations for the effects of the drug. As such, it should be considered that the effects of the inhibitor in an immunocompetent setting could be different from those of constitutive PRMT5 knockdown. In the syngeneic mice growing GL261 tumors, treatment with PRMT5 inhibitor may lead to opposing results on tumor progression. Here, tumor cell invasion, facilitated by cell intrinsic and inflammatory components, is counteracted by decreased cell proliferation. Overall this could result in a lack of survival advantage following PRMT5 inhibitor treatment. Furthermore, NF-κB signaling has been shown to promote resistance to TMZ [34, 298], perhaps explaining why we do not observe any improvement when co-treating with TMZ and perhaps even a trend towards a slightly diminished overall survival. Meanwhile, in the immunocompromised animals bearing U-87 MG tumors, EPZ015666 treatment results in a tumor cell invasion phenotype that is dampened due to lack of cooperating extrinsic immune signals. Athymic mice, such as the ones used in our xenograft experiments, contain severely diminished microglial populations [139]. Coupled with decreased proliferation, this can lead to a notable survival advantage. To test this hypothesis, using the same inducible PRMT5 knockdown experiment proposed previously, it would be important to check that the effect seen in the syngeneic setting is specifically dependent on loss of PRMT5 activity, as opposed to an unwanted side effect of the inhibitor. This hypothesis assumes that GL261 would also shift to a mesenchymal phenotype upon drug treatment, and that the observed immune signature is tumor-promoting as opposed to tumor-suppressive. Overall, these results highlight the importance of also using syngeneic as opposed to only immunocompromised mice for studying tumorigenesis, and highlight that further experiments are needed in order to have a more complete understanding of the effects of PRMT5 inhibition on GBM.

The results of our transcriptional analysis point to a shift in cell identity and proliferative capacity of GBM cells upon PRMT5 inhibition, indicating that PRMT5 may be responsible for maintenance of broad transcriptional programs that define cellular identity. We have preliminary results obtained using TCGA data that show that higher grade gliomas show higher levels of PRMT5 expression than lower grade gliomas, which in turn, exhibit higher expression than normal brain tissue. In light of the results presented here, it will be interesting to use TCGA gene expression data to determine

whether PRMT5 expression levels differ by subtype.

While our results offer important insight into the effects of PRMT5 inhibition, it is critical to address discrepancies with existing literature and understand the caveats of our experimental design. First, the possibility exists that treatment of U-87MG cells with EPZ015666 may also affect the other PRMT family members, contributing to some of the transcriptional effects. Although this is a highly specific inhibitor [44], the concentration used for the gene expression analysis may be high enough to lead to secondary inhibition of the other PRMTs. Another possibility is that since PRMT5 and other PRMT family members often compete for the same substrate, PRMT5 inhibition would result in increased activity of the other enzymes. Such an effect has been described for PRMT1, the main type I PRMT, whose loss results in higher levels of methyl marks deposited by Type II and III enzymes [73]. This type of compensation could have important ramifications. For example, the Bedford lab and others have shown that other PRMT family members, PRMT1, PRMT6 and CARM1 also function as NF-κB coactivators [58, 75, 129, 203]. Therefore, it will be important to determine the levels of the other PRMTs at the protein and mRNA level, as well as their associated methyl marks.

Our results offer a good starting point but remain to be tested in a context that more closely resembles human GBM tumors. While U-87 MG is a widely used human GBM cell line that is easy to grow in culture and transplant intracranially, the limitations of adherent cell lines in resembling *in vivo* tumors are widely appreciated. Furthermore, the incomplete tumor microenvironment these cells develop in upon transplantation into immunocompromised mice hinders the applicability of the results to human tumors. Patient derived gliomaspheres and xenografts, although still lacking the immune system interaction, may complement a mouse syngeneic system and also have been shown to bear resemblance to aspects of human tumors [293, 294]. We are therefore currently extending our studies of PRMT5 loss of function to *in vitro* and *in vivo* patient derived xenograft models of GBM tumors.

While our observation that PRMT5 drives a shift in molecular subtype is based on transcriptome data offering a snapshot in time, with supporting evidence from *in vitro* cellular analyses, it is clear that a multidimensional approach adding protein expression and methylation patterns is necessary for comprehensive subclassification [41]. GBM tumor subtyping based on genetic and epigenetic signatures has been instrumental in revealing and helping to dissect the heterogeneity of this category of tumors. Numer-

ous efforts have been made to replace simple classification schemes based on histological analysis of cellular features with molecular characterizations and biomarkers that can better stratify patients and guide their treatment. However, it has become increasingly clear that tumor heterogeneity adds another layer of complexity and blurs the lines between categories. Single cell sequencing studies have demonstrated that the individual cells that make up a tumor cannot be neatly categorized within the same subgroup, instead, they form a continuum of stemness and proliferation [230]. Perhaps not surprisingly, tumors with higher levels of heterogeneity are associated with poorer clinical outcomes [230], presumably due to their ability to adapt, become resistant, and ultimately repopulate the tumor following therapy. Therefore, while our results demonstrating a shift in cell identity upon PRMT5 inhibition are important for understanding the role of PRMT5 in GBM and the effects of its inhibition at a population level, it is important to remember and appreciate the variability present in patient tumors compared with cell lines.

■ 4.5 Exploring PRMT5 dependencies across tumor cell lines

The gene expression analysis of pathways most correlated with response to EPZ015666 across a panel of tumor cell lines can be used to explore a number of hypotheses. The prominent dependence on splicing and transcription are explored at length in the next section. Comparison of this data with the gene expression signatures following PRMT5 inhibition in U-87 MG cells revealed important similarities. U-87 MG is the most sensitive cell line out of those tested. Therefore, I hypothesized that PRMT5 inhibition in this cell line would result in upregulation of the same pathways that are naturally expressed at higher levels in the more resistant cells, in an effort for these sensitive cells to respond to the drug. Indeed, a broad analysis revealed that resistance was correlated with the Hallmark EMT signature, and Reactome NF-κB signaling. Strikingly, SNAIL expression was a strong predictor of EPZ015666 resistance in the cell line screen (Pearson correlation = 0.59, p < 0.01), hinting that upregulation of SNAIL may be important for determining resistance in U-87 MG cells, as well as in other cancer cell lines.

Using the cell line screen data, we also addressed the relationship between MTAP and PRMT5, which has been recently described [172,198]. Using a pooled shRNA screen, the authors described that loss of MTAP, often found to be codeleted with

CDKN2A, sensitizes cells to shRNA mediated PRMT5 loss [172, 198]. However, due to the mechanism of action of EPZ015666, which is dependent on SAM for cooperative binding, MTAP loss is not entirely predictive of effectiveness of PRMT5 inhibition by this drug. As expected, we also found that lower MTAP expression is not well correlated with EPZ015666 sensitivity (Pearson correlation = 0.30, p = 0.22). This indicates that MTAP levels are not entirely predictive of EPZ015666 response, pointing to the possibility that other factors also influence its effects [172, 198]. Still, the possibility of MTAP as a biomarker for PRMT5 inhibitor response merits further consideration. For example, it is interesting to note that MTAP is deleted in as many as half of GBM tumors [172, 198]. Therefore, it would be interesting to test if this correlation would be stronger when considering only the GBM cell lines in the screen. Furthermore, it is important to note that a drug with a different mechanism of PRMT5 inhibition might show a stronger correlation with MTAP loss, closer to the effects of shRNA-mediated depletion.

We found that resistance to EPZ015666 was modestly correlated with PRMT5 expression levels (Pearson correlation = 0.45, p value = 0.06), and this was higher than any of the other PRMT family members. This supports the specificity of EPZ015666 for PRMT5 but also highlights that PRMT5 levels alone are not a strong predictor of response to this inhibitor. While this analysis is notably limited by being performed on *in vitro* cancer cell lines, it does caution that PRMT5 expression level may not serve as a predictive biomarker for responsiveness to EPZ015666. However, it would be interesting to see whether expression levels of a small set of genes could serve as a predictive signature.

■ 4.6 Assessing the disease relevance of PRMT5-regulated splicing

The most widely cited role for PRMT5 in splicing is as the enzymatic component of the methylosome. This complex, composed of the PRMT5:MEP50 hetero-octamer and the adapter protein pICln, is required for methylation of Sm proteins SmB, SmD1, SmD3 and subsequent SMN mediated assembly of snRNPs in the cytoplasm (Friesen, 2001; Meister, 2001; Brahms, 2000; Friesen, 2002). However, our results as well as others [23, 71, 166, 243] also point to a more specific role for PRMT5 in mediating splicing. The *in vitro* screen for EPZ015666 resistance across a panel of cancer cell lines revealed dependencies on spliceosomal components, as well as categories of genes involved in

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RNA polymerase II (RNAPII)-mediated transcription. These results could be consistent with separate roles for PRMT5 in splicing and transcription, or a novel function for PRMT5 in mediating co-transcriptional splicing. Indeed, PRMT5 has previously been implicated in transcription and it methylates several splicing factors, separate from its role in snRNP assembly in the cytoplasm [26,51,222]. Furthermore, the identification of a subset of detained introns that are regulated by PRMT5 points to a likely role for this methyltransferase in post-transcriptional splicing. As discussed below, these functions may be interrelated and work together to coordinate the effects of PRMT5 on gene expression.

■ 4.6.1 PRMT5 may be involved in co-transcriptional pre-mRNA splicing

Several pieces of evidence point to a function for PRMT5 in RNAPII-mediated transcription. PRMT5 is known to associate with transcription elongation factors SPT4, SPT5 and IWS1, as well as several components of the mediator complex [173,180,189, 285]. SPT4 and SPT5 form a complex that modulates RNAPII processivity and elongation [149] and are involved in mRNA capping [305]. SPT5 is methylated by both PRMT1 and PRMT5 on distinct and overlapping residues, regulating its interaction with RNAPII [173].

The roles for PRMT5 in transcription may be associated to a yet undescribed function in mediating co-transcriptional splicing. Such links are now emerging for a handful of other epigenetic regulators, including PRMT1, and their functions could be described by two mechanisms. The first is a **kinetic model** [192,211], whereby the involvement of PRMT5 in transcriptional elongation may affect alternative splicing. The second **recruitment** model [192] involves interaction between splicing machinery and nucleosomes, facilitated by adapter proteins. Importantly, these two models are not mutually exclusive, and may simply represent different aspects of a more inclusive mechanism [192]. This chromatin adapter model purports that nucleosomes containing specific histone marks are recognized by adapter complexes, which in turn bind splicing factors associated with the RNAPII C-terminal domain (CTD). Other epigenetic factors involved in tumorigenesis have recently been described as linking histone modification, RNAPII mediated transcription, and pre-mRNA splicing [40,121,177,193,256,304]. These observations echo an unfolding story in Type I PRMT biology. In 2011, a study revealed that the CTD of RNAPII is subject to methylation by both PRMT1 and CARM1, resulting in the recruitment of Tudor domain containing protein TDRD3 [257].

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A few years later, the Bedford lab described that TDRD3 complexes with the topoisomerase TOP3B and prevents accumulation of R loops [314]. At the cMYC promoter, PRMT1 and CARM1-mediated asymmetric dimethyl histone modifications are read by TDRD3, which then brings in TOP3B to prevent R loop formation at sites of active transcription [314]. The authors speculate a model in which PRMT1 and CARM1 methylate both histones and the CTD of RNAPII at active promoters. Through recruitment of TDRD3 and TOP3B, R loop accumulation is resolved and transcription progresses [314]. Together with the described roles for CARM1 in splicing [51, 220], it seems plausible that CARM1, and possibly PRMT1, might link chromatin modulation to splicing.

Together, these examples argue for a chromatin code that regulates splicing. It is therefore reasonable to consider that PRMT5, with known roles as an epigenetic modifier, mediates transcription directly through impacting mechanisms governing transcript splicing. The missing piece of the puzzle appears to be an adapter [192], to link together these known roles. These functions might be served by two distinct factors, or one multifunctional complex. I speculate that possible candidates, based on studies in PRMT5 literature, as well as hints in our own data, are SMN, the RNAPII CTD phosphatase FCP1, multifunctional Ewings Sarcoma protein EWSR1, although other candidates, such as CA150, may also exist [51, 111, 244]. Identifying such interactors for PRMT5 may offer new therapeutic targets for modulating certain aspects of PRMT5 function.

A recent report has elegantly expanded the known repertoire of PRMT5 functions by demonstrating an SMN-centric role for PRMT5 in transcriptional termination and the resolution of R loops [323]. R loops are structures composed of an RNA/DNA hybrid and a single stranded DNA that are naturally formed during transcription, and are quickly resolved to prevent DNA damage (reviewed in [258]). Zhao and colleagues found that the RNAPII CTD is methylated competitively on R1810 by PRMT5 and CARM1, and plays a critical role in protecting against extensive R loop formation [323]. The symmetric dimethylation deposited by PRMT5 is read by SMN, which recruits senataxin, an RNA/DNA helicase, important for R loop resolution at sites of transcription termination [259]. Interestingly, Zhao and colleagues speculate that PRMT5 mediated SDMA of R1810 may require a cofactor, which could be FCP1 [323]. Efficient recruitment of senataxin also requires PRMT5-mediated methylation, with SMN therefore bridging the symmetrically dimethylated CTD and senataxin. In line with these ob-

servations, R1810 mutation or knockdown of either PRMT5 or SMN resulted in DNA damage, likely due to the genomic instability resulting from inefficient resolution of R loops [323]. Linking these observations to splicing, the authors mention in passing that R1810 mutants show splicing defects, although no data is provided. Furthermore, R loops slow down elongation (reviewed in [236]) and R loop formation hinders spliceosome assembly [282], underscoring the importance of resolving this structure for the maintenance of splicing fidelity. Since we observe increases in certain DNA damage markers after PRMT5 inhibition in U-87 MG cells, it would be interesting to determine whether there is also accumulation of senataxin, pointing to the importance of PRMT5 for R loop resolution in our system.

SMN is known to self-aggregate and serve as a docking site for other factors. Therefore, just as it can bind to CTD and also to symmetrically dimethylated senataxin, it is plausible that SMN could bind to similarly modified nucleosomes. Early on, SMN was described to be localized to nuclear Cajal bodies in an SDMA-dependent manner [25]. These nuclear structures may be important not only to splicing fidelity but to genome organization [296]. Understanding how PRMT5 may function as part of these structures may reveal further complexities of PRMT5 mediated gene regulation.

Other proteins that would be interesting to explore for possible roles linking PRMT5 and co-transcriptional splicing are the RNAPII CTD phosphatase FCP1, and the Ewings sarcoma protein (EWSR1). FCP1 is required for transcriptional elongation, is a substrate for PRMT5, and also interacts with SmB and U1-70K, a U1 snRNP specific protein [6, 184]. PRMT5, while bound by FCP1, was capable of methylating histones H4 and H2A [6]. However, the functional consequences of FCP1 methylation are not yet well understood. EWSR1 is a PRMT5 target [26] that links RNAPII to splicing via the transcription factor YB-1 [46]. Recently, EWSR1 has been descried to interact with FUS/TLS, another PRMT5 substrate [26], in regulating transcription and mRNA processing [194]. EWSR1 offers an interesting target for further study because several gene signatures related to its function were observed in our GSEA analysis following PRMT5 inhibition in U-87 MG cells.

■ 4.6.2 Detained introns regulated by PRMT5 point to a role in post-transcriptional splicing

Our colleagues in the Koch Institute have recently described detained introns (DIs) as a novel class of introns that are present in polyadenylated, and therefore otherwise fully transcribed mRNA transcripts [29]. As opposed to the more widely recognized class of retained introns, which occur as a result of alternative splicing, DIs are present in both constitutive and alternatively spliced introns. Retained introns are the result of alternative splicing events and are maintained in the mature mRNA transcript, which is then exported to the cytoplasm. Subsequent translation either results in a protein product, or degradation via nonsense mediated decay (NMD). Meanwhile, persistence of DIs in the mRNA transcript restricts them in the nucleus. Their nuclear localization protects them from NMD, and they do not increase in abundance when NMD is impaired [29]. Importantly, DI-containing genes can produce both DI-containing transcripts and productive, coding isoforms. Of these, only the latter result in protein products. From our data, it is clear that PRMT5 controls the excision of some DIs, while others, found to be overlapping between the EPZ015666 and vehicle treated cells, are controlled independently of PRMT5. Similarly, Clk kinase, which phosphorylates SR protein involved in splicing, also controls a subset of DIs [29]. Some of the Clk-responsive DIs are also regulated by DNA damage, indicating that DIs can be regulated in response to stress signals [29]. This helps formulate the hypothesis that PRMT5 might also respond to cellular stress signals via regulation of DI splicing.

While the majority of splicing occurs co-transcriptionally, an estimated 15-20% occurs post-transcriptionally [109]. Underlining its regulatory nature, it appears that alternative splicing largely takes place post-transcriptionally [209, 280, 289]. Interestingly, using single molecule imaging to visualize splicing reactions, Vargas and colleagues reported that uncoupling transcription from splicing has the potential to regulate specific introns in a transcript independently of other introns, which are removed co-transcriptionally [289]. Others have reported that the post-transcriptional spliceosome is present in nuclear speckles [109], inter-chromatin structures which have long been known to contain splicing components (reviewed in [260]). Previously, most DIs were found to be spliced post-transcriptionally, with a subset occurring co-transcriptionally [29]. Indeed, hnRNPs and SR proteins, which are involved in splicing and are found in nuclear speckles, can also be modified by PRMT5 [26, 283]. While this remains to be verified in our system, it does appear possible that PRMT5 could be involved in regulating

post-transcriptional splicing.

A recent report may help in building a more detailed model of the PRMT5's involvement in splicing. In *Arabidopsis thaliana*, Deng and colleagues found that AtPRMT5-mediated AtSm protein methylation is necessary for assembling a non-snRNP protein complex, Prp19, into the U4/U6.U5 tri-snRNP, to form the active spliceosome. In atprmt5 mutants, recruitment of Prp19 could be rescued by gain-of-function mutations in pre-mRNA processing factor 8 (prp8), a protein associated with the U5 snRNP [71]. They present a structural hypothesis, whereby decreased Sm protein methylation in atprmt5 mutants changes the stability of the spliceosome and therefore the recruitment of the Prp19 complex. Mutant Prp8 may slightly change the spliceosomal conformation, overcoming the effects of atprmt5 mutation. Importantly, in line with our data, they also observe increased intron retention and weaker 5' splice sites in atprmt5 mutants. While these results offer an intriguing avenue for further exploring the mechanism of PRMT5-dependent splicing, it remains to be seen whether this proposed mechanism holds true in mammals. It is interesting to note that in a human breast cancer cell line, PRP8 depletion also results in retention of introns with weak 5' splice sites [306]. In light of these data, one could verify whether loss of Prp8, or Prp19 components could phenocopy the splicing defects and patterns observed upon PRMT5 inhibition, and whether PRP8 overexpression could rescue these effects. It would also be interesting to test whether PRP8 overexpression could rescue the proliferative defects observed upon PRMT5 loss or inhibition or how this relates to tumorigenesis *in vivo*.

We have identified a role for PRMT5 in regulating splicing of detained introns in GBM cells. This agrees with what had been identified previously with PRMT5 loss of function in neuron progenitor cells and mouse embryonic fibroblasts [23]. However, while these introns were previously classified as retained introns, we believe that most of them are more accurately classified as detained introns. It would be important to verify whether the same splicing patterns observed upon PRMT5 inhibition with EPZ015666 hold true with PRMT5 knockdown. Notably, PRMT5 knockout using CRISPR/Cas9 has not been successful in our hands, and has been noted to be lethal [198], underscoring the importance of PRMT5 for cell viability.

As a critical next step, we will determine the overlap between differentially expressed genes and DI containing genes and analyze the relative levels of DI containing transcripts compared to their coding counterparts in response to EPZ015666. Thus far, it appears that while DI containing genes compose a significant fraction of the differen-

tially expressed genes, not surprisingly, there are groups of non-DI containing genes that are modulated following drug treatment. This represents splicing independent mechanisms through which PRMT5 modulates gene expression, possibly directly through its effects as a co-transcriptional regulator or repressor, or indirectly, by affecting growth factor signaling pathways. It will be important to identify whether the genes that are regulated by intron detention upon PRMT5 inhibition belong to certain functional categories. This would help determine whether one of the ways that PRMT5 normally impacts gene expression impinging on a particular pathway or cellular process is by regulating the levels of DIs. However, it is possible that a small subset of pathways will not become apparent, and instead the functional categories will be more reflective of a cross section of PRTM5 functions. Such a result could be expected, given that PRMT5 could use a number of different regulatory roles to impact the same biological function, such as altering transcriptional activity, direct interaction with signaling molecules, and regulation of alternative splicing events.

■ 4.6.3 Building an integrated model for PRMT5 activity

Intron retention is a possible mechanism by which gene expression could be downregulated, independent of changes in transcription [78]. Similarly, DIs could offer a mechanism of regulation whereby DI-containing genes would be contained in the nucleus, unable to be translated. In a study across 16 different cancer types, intron retention was found to be a more prevalent mechanism of regulating gene expression in tumor tissues compared to normal controls [78]. It is perhaps unsurprising then, that in response to PRMT5 inhibition, which undoubtedly perturbs homeostasis, tumor cells with higher expression levels of splicing factors are more resistant to this external stressor. Our results are important in teasing apart the most pertinent aspects of PRMT5 biology for tumorigenesis, perhaps pointing to a common regulatory mechanism that many tumor types would use to respond to PRMT5 inhibition.

Our data demonstrate that PRMT5 inhibition results in a subset of differentially expressed genes, of which a proportion contain DIs. This implies that PRMT5 affects gene expression both through splicing and by modulating transcription of other genes, either directly or indirectly by affecting other cellular processes, such as signaling. If the chromatin adapter model holds true for PRMT5, it might uncover a complex web of transcriptional regulation which PRMT5 helps to fine tune.

At one level, it is possible that the alternative splicing facilitated by PRMT5 is related to the chromatin structure surrounding the affected genes. Potentially, certain histone marks deposited by PRMT5, but not others, could signal for regulation of gene expression by splicing. Additionally, by methylating the RNAPII CTD or different elongation factors, PRMT5 might affect the rate of transcription, which could in turn impact splicing, or may represent an independent mechanism of transcriptional regulation. Finally, at yet another layer of regulation, PRMT5 dependent methylation may regulate post-transcriptional splicing, affecting the binding strength of certain RNA binding proteins, thereby modulating their effects and mediating excision or inclusion of certain DIs. It is possible that during transcription, PRMT5 marks certain DIs for inclusion or exclusion, and this process is completed post-transcriptionally [122]. By regulating DIs, PRMT5 regulates a specific pool of almost fully processed transcripts that are poised for nuclear export and translation in response to specific stresses. It is also plausible that PRMT5 exerts effects on splicing by linking it to mRNA export [122]. Altogether, it is perhaps unsurprising that PRMT5, known to be a pleiotropic protein, links together both long and short term mechanisms of regulating gene expression, via its involvement as a co-transcriptional regulator and splicing mediator, respectively.

■ 4.6.4 Further dissecting the role of PRMT5 in splicing

Our data point to an important role for PRMT5 in modulating gene expression, partially through splicing. These results offer interesting insights into PRMT5 biology and exciting avenues for further exploring the mechanisms behind its functions. While our cell line screen data reveal that high levels of splicing and transcriptional components are correlated to resistance to PRMT5 inhibition, it is possible that this pattern of gene expression mediates cross-resistance to several drugs. Although preliminary results indicate that this is not the case, and the effect is specific to PRMT5 inhibition, we could use CCLE data [16] on responsiveness to different drugs to test the determinants of sensitivity or resistance. Building on this, and since we have TCGA data showing that higher levels of PRMT5 expression are associated with higher grade versus lower grade gliomas (data not shown), it would be interesting to interrogate whether the role of PRMT5 in splicing is relevant to human gliomas. To do this, one could use poly(A) selected RNAseq data from TCGA to look at differences in splicing patterns in tumors expressing high versus low levels of PRMT5, as has been previously reported in breast cancer [78] and clear cell renal cell carcinoma [256].

It will first be important to assess the contribution of PRMT5-dependent Sm protein methylation to PRMT5 mediated splicing of DIs. To do this in a manner that is specific to PRMT5 activity and does not affect snRNP assembly is any other way, one option would be to generate cell lines expressing Sm proteins (SmB, SmD1, SmD3) containing mutations of arginine residues specifically methylated by PRMT5 substrates. We could then assess gene expression and DI changes for these mutants. Next, we could use nuclear and cytoplasmic fractionations to verify that DI-containing transcripts are in fact restricted to the nucleus. To begin to tease apart the mechanism by which PRMT5 modulates splicing, we can also perform nuclear subfractionations. This would allow us to verify the levels of PRMT5 that are associated with the chromatin versus non-chromatin soluble fraction, which is known to be associated with nuclear speckle components such as SR proteins [228]. Given its known functions as a histone modifier, I expect to find PRMT5 in the insoluble chromatin-associated fraction. Its presence together with components of the nuclear speckles would lend weight to the hypothesis of its effects in mediating post-transcriptional splicing. It would also be informative to test the temporal and spatial decoupling of splicing mediated by PRMT5 to better understand what proportion of PRMT5-mediated splicing changes are likely to occur through co-transcriptional versus post-transcriptional splicing mechanisms.

■ 4.7 Conclusion

The field of PRMT5 biology has been rapidly expanding in the past few years and we are on the cusp of exciting advances describing the multifaceted roles of PRMT5 in numerous cancers. With the therapeutic relevance of targeting this protein becoming increasingly apparent, our work demonstrates an exciting advancement in linking the role of PRMT5 in glioblastoma to a complex mechanism for the regulation of gene expression.

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