



Understanding the requirement of PRMT5 splicing regulation in Merkel cell carcinoma.

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HARVARD UNIVERSITY
Graduate School of Arts and Sciences



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Merkel cell carcinoma*

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Understanding the requirement of PRMT5 splicing regulation in Merkel cell carcinoma.

A DISSERTATION PRESENTED

BY

CAMILLE H. CUSHMAN

TO

THE DIVISION OF MEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE SUBJECT OF

VIROLOGY

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Understanding the requirement of PRMT5 splicing regulation in Merkel cell carcinoma.

ABSTRACT

Merkel cell carcinoma (MCC) is an aggressive, highly metastatic neuroendocrine skin cancer. Immune checkpoint therapies have greatly improved the overall survival rate of patients with late-stage disease, however patients with resistant tumors have very limited alternative treatment options. In this dissertation, we investigate the molecular mechanisms of MCC growth and survival to identify new avenues of targeted therapeutics for MCC.

Previous efforts to characterize the molecular features of MCC have identified two distinct subtypes: polyomavirus-associated MCC (MCCP) and non-viral MCC (MCCN). MCCP is caused from integrated Merkel cell polyomavirus (MCV) DNA while MCCN tumors are caused from UV damage. Recent work showed that MCV small tumor antigen (ST) regulates transcription by binding with the proto-oncogene L-MYC and the TIP60/P400 chromatin remodeling complex (the SLaP complex). In this dissertation, we extend these findings by showing that multiple TIP60 isoforms bind to the SLaP complex and that chromatin binding of SLaP is correlated with H2A.Z acetylation, a histone variant mark associated with gene activation. These findings provide further support for a model where ST recruits the TIP60/P400 complex to activate specific gene expression via chromatin remodeling at the transcriptional start site of L-MYC target genes.

Next, we show that PRMT5, a transcriptional target of the SLaP complex, is required for growth of MCC cells. PRMT5 is an arginine methyltransferase with diverse cellular targets, including the well-described tumor suppressor p53. We found that PRMT5 loss induces p53 activity, cell cycle arrest, and apoptosis; however, we show that p53 activity is not required for cell viability loss. Using unbiased approaches, we identify mRNA processing as a critical pathway dysregulated by PRMT5 inhibition in MCC. We show that PRMT5 is required for splicing of detained introns in cell cycle and DNA damage genes. We further investigate the role of PRMT5 in regulating the DNA damage response and find that PRMT5 is required for the ATR/CHK1 response and genome stability. Overall, we take a molecular approach to identify PRMT5 as a potential therapeutic target for MCC and characterize the role of PRMT5 in regulating both mRNA splicing and the response to DNA damage.

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AUTHOR CONTRIBUTIONS FOR CHAPTER 1

In this introductory chapter, the sections “1.1.1: History of MCC” and “1.2: Investigating the molecular mechanisms of MCC growth reveals distinct but convergent etiologies.” and the Figures 1.1 and 1.2 were adapted from a published review article co-authored with Mona M. Ahmed and James A. DeCaprio¹. Figure 1.1 was adapted from a figure originally generated by Mona M. Ahmed and Figure 1.2 was adapted from a figure originally generated by Camille H. Cushman.

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1

Introduction

1.1 MERKEL CELL CARCINOMA (MCC) IS AN AGGRESSIVE NEUROENDOCRINE CANCER WITH LIMITED TREATMENT OPTIONS.

1.1.1 HISTORY OF MCC

Merkel cell carcinoma (MCC) was first described in 1972 by Cyril Toker as trabecular carcinoma of the skin, a reference to the column-like appearance of the tumor cells² (reviewed in³). Electron microscopy experiments in a number of these tumors

revealed the presence of dense-core secretory granules⁴, membrane-bound repositories of proteases and hormones involved in the regulated secretory pathway specific to endocrine or neuroendocrine cells^{5,6,7}. The only cells in the skin with dense-core granules are Merkel cells which is where MCC acquired its modern name.

Merkel cells are a unique and relatively rare neuroendocrine skin cell involved in signal transduction of gentle touch stimuli⁸. They are a terminally differentiated, non-dividing cell thought to originate from epithelial progenitor cells in the skin epidermis. Like Merkel cells, MCC cells commonly express synaptophysin, chromogranin A, neuron-specific enolase, and cytokeratin 20 (CK20)^{9,10}; reviewed in¹¹. Despite the similarities in staining and appearance, there is debate in the field as to whether MCC tumors originate from Merkel cells. A few arguments against the Merkel cell origin theory include: the finding that the location of Merkel cells is within the epidermis while MCCs are typically found in the dermis; the divergent patterns of CK20 staining; the presence of additional markers in MCC cells not found in Merkel cells; and the lack of detection of any benign Merkel cell tumors or even any proliferating Merkel cells (reviewed in^{12,13}). Despite this debate, the name Merkel cell carcinoma has endured and our understanding of both the underlying etiology and the clinical features of this disease have worked to measurably improve disease detection and patient outcomes since its original characterization.

1.1.2 CLINICAL FEATURES OF MCC

MCC presents in the clinic as a rapidly growing, flesh-to-red colored firm lump (reviewed in^{11,14}). The acronym AEIOU has been used to describe the clinical presentation of MCC, where “A” stands for asymptomatic, as in the tumor is painless; “E” stands for expanding rapidly; “I” stands for immune suppression; “O” stands for older

than age 50; and “U” stands for UV-exposed, as MCC is more common among fair-skinned individuals on UV-exposed skin¹⁵. The incidence of MCC in the U.S. is low but has risen over the past few decades, from 0.55 per 100,000 men and 0.28 per 100,000 women in 1997 to 1.03 and 0.45 per 100,000 in 2016 in men and women respectively¹⁶. This increase may be due in part due to an increase in an aging, sun-exposed population or due to increased awareness of MCC among clinicians and an improved ability to distinguish MCC from other neuroendocrine cancers using immunohistochemistry staining.

Historically, MCC was considered a highly metastatic cancer. In 1999, it was reported that over half of MCC patients develop regional lymph node metastases¹⁷. Despite progress in MCC diagnostic methods and treatment options, MCC is still today considered to be frequently metastatic with the mean latency between primary diagnosis to systemic metastasis being approximately 2.1 years¹⁸. Compared to cutaneous malignant melanoma, another malignant skin cancer, MCC has a higher proportion of patients with distant metastatic disease at time of initial diagnosis¹⁹. In the next section, we will review the MCC staging protocols and current treatment options for patients with local or metastatic MCC.

1.1.3 CURRENT TREATMENT PROTOCOLS AND STAGING

In 2016, the staging protocols for MCC were revised for the 8th edition of the American Joint Committee on Cancer (AJCC). By analyzing 9,387 MCC cases, Harms et al.²⁰ re-defined MCC staging guidelines for the 5 major cancer stages (Stage 0-IV, with Stage II and Stage III being additionally divided into A and B groups) based on the TNM (Tumor, Node, Metastasis) Classification. Briefly, stages 0, I, IIA, and IIB describe tumors in increasing size with no regional lymph node metastases nor

any distant metastases. Stage III includes tumors of any size with any regional lymph node metastasis. This clinical stage can be divided into two pathological stages: stage IIIA includes primary tumors of any size with a clinically occult regional lymph node metastasis OR an undetectable primary tumor with a clinically detected regional lymph node metastasis; stage IIIB includes detectable tumors of any size with a clinically detected regional lymph node metastasis, including in-transit metastases with or without a lymph node metastasis. Stage IV includes any cancer with a distant metastasis, no matter primary tumor size or presence/absence of lymph node metastases.

These stages were defined in part based on patient prognosis. Based on the Harms et al. analysis from 2016²⁰, the 5-year overall survival rate of patients with pathological stage I disease was 62.8%; stage IIA was 54.6%; stage IIB was 34.8%; stage IIIA was 40.3%; stage IIIB was 26.8%; and stage IV was 13.5%. These statistics highlight the need for improved treatment strategies, particularly for patients with later stage disease, although notably these statistics were determined prior to the approval in 2017 of immune checkpoint therapies as a treatment for MCC (reviewed in²¹). Current treatment protocols as outlined by the National Comprehensive Cancer Network (NCCN: NCCN Guidelines Version 2.2022 for Merkel Cell Carcinoma) include surgical excision of the primary tumor with adjuvant radiation therapy to the primary site in most cases. If a lymph node metastasis is detected (and even in some high risk cases when it is not), radiation therapy to the nodal basin is recommended along with clinical trial adjuvant therapy, systemic adjuvant chemotherapy, or neoadjuvant immunotherapy. Systemic chemotherapy includes cisplatin or carboplatin $\pm$ etoposide, although this treatment is generally not recommended unless “clinical judgement dictates” as retrospective data suggests that these chemotherapy treatments are not durable.

For metastatic disease, the treatment options are limited, although the advent of immunotherapies has greatly impacted survival for patients with late stage MCC. In addition to surgery and radiation, the NCCN recommends a clinical trial and the preferred interventions are anti-PD-L1 and anti-PD-1 immune checkpoint therapies including avelumab, pembrolizumab, and nivolumab. In the next section, we will briefly discuss the impact of the advancement of immunotherapies on MCC patient responses.

IMMUNE CHECKPOINT THERAPY: ADVANCES AND ALTERNATIVES

Immune checkpoint therapy has proved effective in numerous clinical trials for many advanced or metastatic cancers including melanoma, non-small-cell lung carcinoma, head and neck squamous cell carcinoma, Hodgkin lymphoma, urothelial carcinoma, small cell lung cancer, and Merkel cell carcinoma (reviewed in^{22,23}). The theory behind immune checkpoint therapies centers around the idea of re-engaging the immune system to recognize cancer cells as “non-self” and target them for cytotoxic T-cell killing (reviewed in²³). Pioneering work by James Allison and Tasuku Honjo identified cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death molecule-1 (PD-1) respectively and showed that blocking binding of CTLA-4 and PD-1 to their respective ligands could break immune tolerance of the tumor cells. These discoveries paved the way to the development of immune checkpoint blockade therapy: using antibodies specific to CTLA-4, PD-1, PD-L1 (ligand of PD-1) to block the T cell inhibitory function of these molecules.

The advent of immune checkpoint therapies has dramatically changed the prognosis for many patients with late stage or metastatic MCC, and treatment with avelumab (anti-PD-L1) or pembrolizumab (anti-PD-1) is currently recommended as the first-

line treatment for these patients, although there are ongoing clinical trials for additional checkpoint blockade therapies such as nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) (reviewed in²⁴). Recently, in March of 2023, another anti-PD-1 drug retifanlimab-dlwr was approved based on promising results from clinical trial PODIUM-201 (NCT03599713). 50-70% of late stage MCC patients respond to anti-PD-1/PD-L1 therapies as a first-line treatment, and of the patients who initially respond to these therapies, 70-80% have a response durability at 2 years. In comparison, only 5% of late stage MCC responders to conventional chemotherapies continue to have a treatment benefit at 2 years.

Although immune checkpoint therapy has been life-changing for many patients with late stage MCC, approximately 50% of these MCC cases have either intrinsic or acquired resistance to immune checkpoint therapies (reviewed in²⁴). These therapies are also not suitable for the proportion of patients with pre-existing severe autoimmune disease or who have received a solid organ transplant and have ongoing immunosuppression. This is a real issue for many MCC patients, as MCC is more common amongst immunosuppressed individuals. The alternatives to immune checkpoint therapy for late stage MCC patients is limited. At the time of writing, there are a number of ongoing clinical trials for targeted therapies including domatinostat (a histone deacetylase inhibitor), Lu 177 dotatate (a somatostatin analog), and KRT-232 (an MDM2 inhibitor). The utility of targeted therapies depends on our understanding of the underlying molecular biology of the disease, often on a patient-specific level. Continued efforts to identify targetable features of MCC is necessary to improve the alternative therapies available for the significant proportion of MCC patients with refractory late stage disease.

1.2 INVESTIGATING THE MOLECULAR MECHANISMS OF MCC GROWTH REVEALS DISTINCT BUT CONVERGENT ETIOLOGIES.

As discussed above, much progress has been made clinically in the treatment of MCC especially with the advent of immunotherapies. However, there remains a large proportion of patients who have contraindications to current treatments or who experience refractory recurrence following treatment. Therefore, there is a need for continued efforts to better understand this disease to identify additional treatment strategies. Here, we will summarize the progress made to identify the molecular mechanisms underpinning MCC growth and development since Toker's initial characterization.

POLYOMAVIRUS-ASSOCIATED MCC (MCCP) AND NONVIRAL MCC (MCCN)

A major discovery in the advancement of our understanding of MCC was the identification of polyomavirus DNA clonally integrated into the genome of many MCC tumors. Using a digital transcriptome subtraction method, Huichen Feng along with Masahiro Shuda in the Chang-Moore lab identified and then named this novel polyomavirus Merkel cell polyomavirus (MCV or MCPyV)²⁵. Prior to the identification of MCV, MCC was classified into "Classic" and "Variant" groups, and analysis of the gene expression patterns suggested there may be unique etiologies between these MCC types²⁶. Additionally, the increased incidence of MCC among the immunosuppressed^{27,17} suggested to Feng et al. that there may be an infectious origin to at least one type of MCC. Since 2008, many groups have worked to better characterize the biology of this novel polyomavirus and clarify the role of viral proteins in oncogenesis.

MERKEL CELL POLYOMAVIRUS (MCV)

MCV is a highly prevalent virus that causes a persistent, lifelong, and generally innocuous infection in most people (reviewed in²⁸). Like other polyomaviruses, MCV is a small, non-enveloped, double-stranded DNA virus with a chromatinized genome that is a little over 5 kb (Figure 1.1 A). The genome can be divided into two major regions: the early region, which encodes for at least two unique viral tumor antigens (T antigens) known as small T (ST) and large T (LT); and the late region, which contains genes for the structural capsid proteins viral protein 1 (VP1) and viral protein 2 (VP2) (Figure 1.1B). Between these two regions lies a non-coding control region (NCCR) that serves as a bidirectional promoter and contains the viral origin of replication.

Upon viral infection, transcription of the early region is initiated leading to expression of the T antigens. LT and ST are splice variants of one another; ST is expressed from transcriptional read-through of the splice site used to produce LT transcripts. There are two additional gene products in the early region that have been described: 57kT, another splice variant of LT; and ALTO, a protein produced from an alternative open reading frame found within the second exon of LT. The functions of these additional proteins during the viral life cycle or in the context of MCC are relatively unknown.

The late gene region of MCV encodes for the capsid proteins, viral protein 1 (VP1) and viral protein 2 (VP2). These structural proteins are expressed after viral DNA replication has been initiated and cooperate to promote viral entry^{29,30}. In addition, the late region also encodes a viral microRNA (miRNA) that regulates early gene expression. This 22-nucleotide sequence is complementary to LT just downstream from the region encoding the pRB-binding motif (nucleotides 1217-1238). The MCV

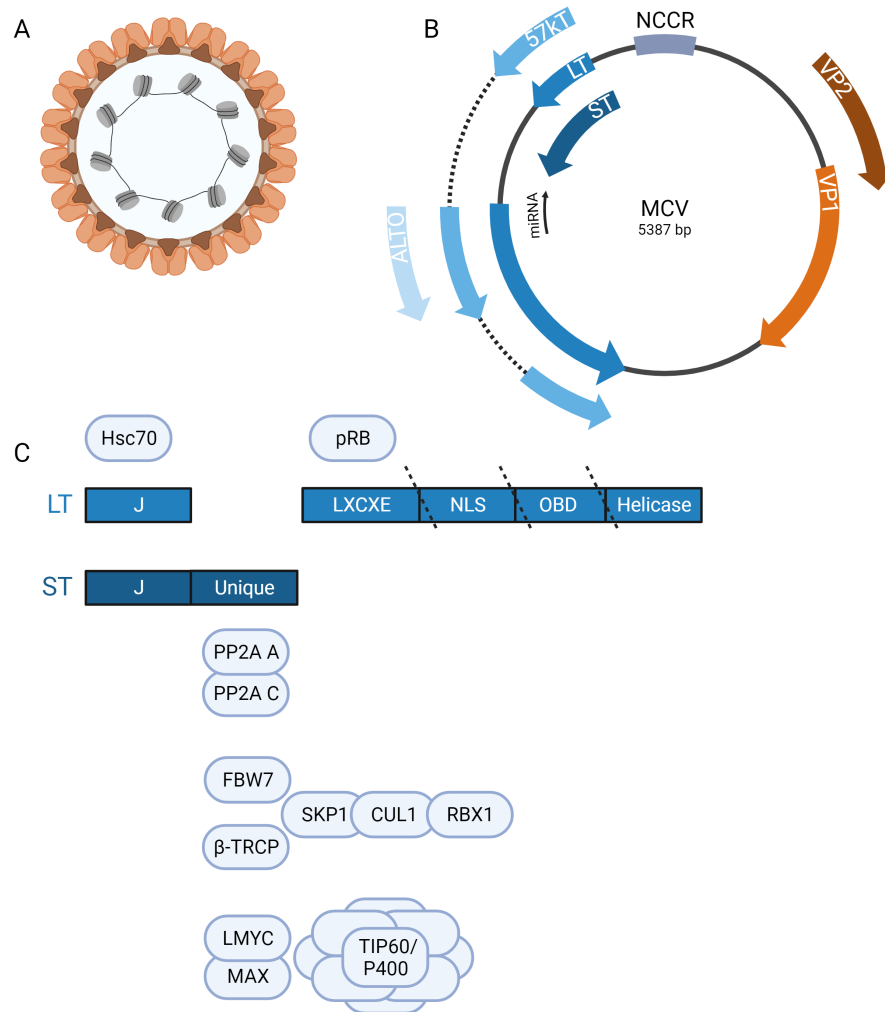


Figure 1.1: Merkel cell polyomavirus (MCV) genome architecture and T antigen interactions. **A.** Cartoon of a MCV virion cross-section depicting the non-enveloped capsid and chromatinized genome. **B.** Map of MCV genome coding regions including the non-coding control region (NCCR), the early region encoding the viral T antigens, and the late region encoding the capsid proteins. **C.** T antigen domains and the respective host protein binding partners that have been previously identified. Dashed lines on LT cartoon indicate approximate LT truncation mutations that have been previously identified in MCC. Created with BioRender.com.

miRNA is expressed at low levels in about half of MCCP tumors but its contribution to MCC tumorigenesis is unclear.

As the coding capacity is limited by the genome size, the MCV genome does not contain the polymerases necessary for gene expression and genome replication. Instead, these small viruses rely entirely on host DNA and RNA polymerases. The function of the T antigens therefore is to promote conditions within the host cell favorable for DNA replication. Having a double-stranded DNA genome, polyomaviruses can mimic host cell biology by forming a mini-chromosome of the viral genome wrapped around host cell histones. This chromatinized genome is an important part of polyomavirus biology and the genome is in fact encapsulated into the mature virions in a chromatinized form. Although the mechanisms have not been elucidated, it seems apparent that maintaining an open, active chromatin state along the viral genome is necessary for viral protein expression.

The activities of LT are thought to play a crucial role in driving the viral life cycle with the activities of ST enhancing LT function. The protein sequence of LT contains a number of unique domains (Figure 1.1C). At the C-terminal end or unique region of LT, there is a helicase domain and an origin binding domain (OBD). These domains are important for initiating viral DNA replication and promoting synthesis of the viral genome. The LT sequence also contains a nuclear localization signal and a pRB-binding domain. The N terminal sequence of LT is shared with ST and contains a DnaJ domain (or J domain) known for binding to heat shock factor proteins. This domain has been implicated in regulating a number of stages of the polyomavirus life cycle, including DNA replication and capsid formation^{28,31,32}. These studies also found that an intact J domain is required for the pRB binding activity of LT and is therefore likely important for oncogenesis in MCCP as well.

The canonical understanding of polyomavirus T antigen activity is that LT is the primary oncoprotein while ST plays a secondary role and is not sufficient to drive transformation. MCV differs from the norm in that MCV ST expression alone is sufficient to induce cellular transformation in Rat1 cells³³ and tumor formation in p53-null transgenic mice³⁴, suggesting that MCV ST has unique functions. Like other polyomaviruses including SV40, JCPyV, and BKPyV, MCV ST can displace certain PP2A subunits to inhibit specific PP2A activities³⁵ (Figure 1.1C). PP2A, or protein phosphatase 2A, is a family of holoenzymes with Ser/Thr phosphatase activity composed of a structural subunit (PP2A A), a catalytic subunit (PP2A C), and a variable subunit (PP2A B) that distinguishes PP2A binding partners (reviewed in³⁶). SV40 ST can bind to PP2A A α subunits and prevent the assembly of PP2A complexes containing B55 α , B56 α , or B56 ϵ subunits. MCV ST binds to PP2A A α and A β and has only been shown to inhibit complex assembly with the B56 α subunit³⁷. PP2A binding is thought to be critical for the oncogenic activities of SV40 ST^{38,39} but is dispensable for MCV ST-mediated cellular transformation³³. In accordance with this understanding, SV40 ST prevents PP2A-mediated dephosphorylation of AKT while MCV ST does not⁴⁰. From these findings, it seems likely that the critical oncogenic activities of MCV ST lie with alternative protein interactions.

To gain a better understanding of how ST contributes to oncogenesis, many molecular studies in the field have worked to identify the binding partners of ST and characterize the significance of these interactions. One way in which MCV ST may contribute to viral replication and oncogenesis is through stabilizing MCV LT expression, a phenomenon that has been documented by multiple groups^{40,41}. Kwun et al. suggest that ST stabilizes LT by binding and inhibiting the SCF(FBW7) ubiquitin ligase complex, which they found targets LT for degradation (Figure 1.1C). Kwun et al.

mapped the residues in ST required for LT stabilization and found them to be amino acids 91-95, which they named the LT stabilization domain or LSD⁴⁰ (Figure 1.2). In addition to SCF(FBW7), Kwun et al. also found that ST binds to SCF(B-TRCP) in an LSD domain-specific manner and show that the expression of both SCF(FBW7) and SCF(B-TRCP) targets such as c-MYC, Cyclin E, and PLK2 are stabilized with WT ST expression. The LSD domain has since been linked to additional oncogenic activities of ST, including activation of non-canonical NF- κ B signaling⁴², and upregulation of matrix metalloproteinase expression⁴³. However, the interaction between SCF complexes and the T antigens has been called into question. Dye et al. noted that neither MCV ST nor MCV LT contain the canonical FBW7 phospho-degron, and they were unable to reproduce the finding that MCV ST binds to either SCF(FBW7) or SCF(B-TRCP)⁴¹. This study did support the finding that the LSD domain of ST stabilizes LT, suggesting that ST may promote LT expression through an alternative mechanism. Further work is necessary to explain the discrepancy between these findings and to clarify the function of the LSD domain.

To identify additional binding partners of ST, a group in the DeCaprio lab performed an immunoprecipitation of MCV T antigens and identified associated proteins by mass spectrometry⁴⁸. This study found that MCV ST uniquely binds to the oncogenic transcription factor L-MYC (*MYCL1* gene) and its heterodimerization partner MAX along with all the components of the transcriptional regulatory complex known as the TIP60/P400 complex (NuA4 complex in yeast) (Figure 1.1). Here, we will refer to the ST, LMYC, and P400 complex as the SLaP complex. Further interrogation of these data showed that ST expression induces binding between L-MYC/MAX and the TIP60/P400 complex and that two glutamic acid residues (E86, E87) are required for SLaP complex formation. By threading the sequence of MCV ST onto the crys-

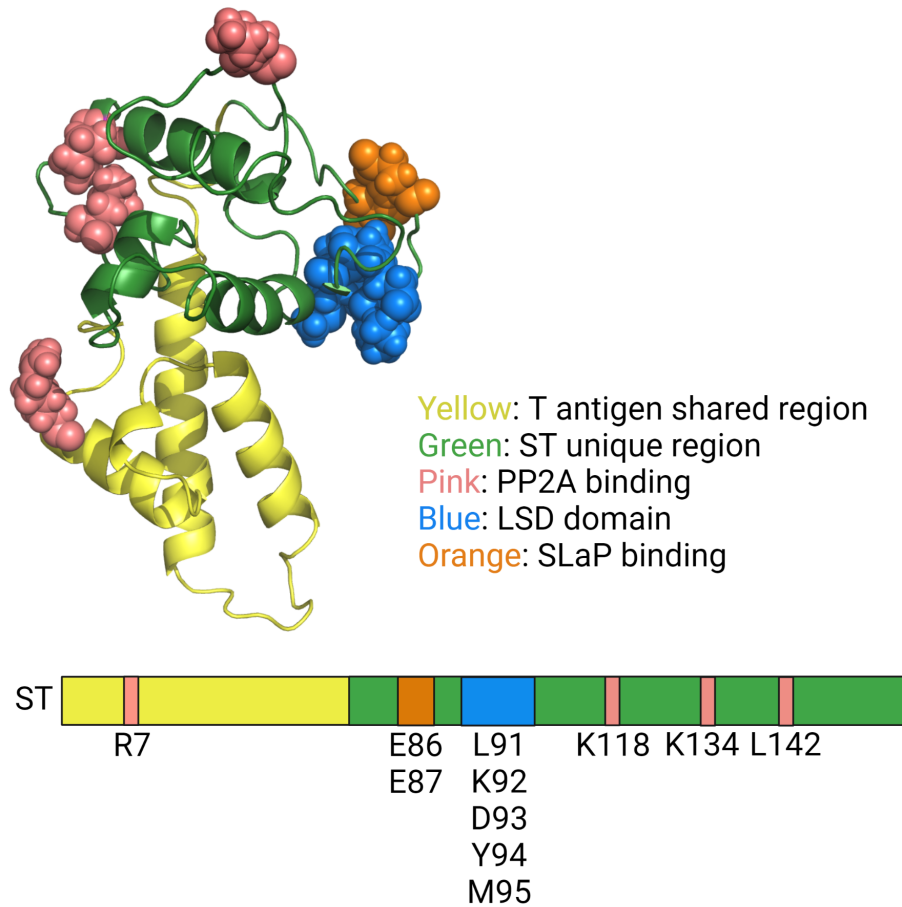


Figure 1.2: Predicted structure and domains of MCV ST. The structure of MCV ST was modeled by threading the MCV ST sequence onto the SV40 ST crystal structure (PDB ID: 2PF4) using the I-TASSER server^{44,45,46} and regions of interest were highlighted both on the structure and the gene diagram below. The structure was visualized in PyMol⁴⁷ and the diagram was created with BioRender.com.

tal structure of SV40 ST using the I-TASSER server^{44,45,46}, we can see that these residues are predicted to be located proximal to the LSD domain (Figure 1.2). Notably, Cheng et al. observed that substitution or deletion of the LSD domain reduced SLaP complex formation. Further work is necessary to fully elucidate the structure and functions of ST in both the context of viral replication and oncogenesis.

1.2.1 DISTINGUISHING FEATURES BETWEEN MCCP AND MCCN

The discovery of MCV changed the field of MCC research, and much work has been done since to better characterize the molecular and clinical features of polyomavirus-associated MCC (MCCP) and nonviral MCC (MCCN). Clinically, MCCP and MCCN tumors present very similarly but can be distinguished using tests designed to directly or indirectly detect the T antigens such as T antigen-specific immunohistochemical staining of tumor sections or the AMERK test, a measure of T antigen-specific antibodies⁴⁹. Healthy individuals rarely have antibodies to the T antigens but a majority of MCCP patients do have serum antibodies to LT and ST, therefore T antigen antibody titers are considered a reliable biomarker to monitor cancer remission and recurrence⁴⁹. Following treatment and during cancer remission, T antigen antibody titers dramatically decrease and remain at low levels. An increase in antibody titers following treatment suggests cancer recurrence and prompts a search for disease.

The rise of sequencing technologies in recent years has highlighted another striking molecular difference between MCCP and MCCN tumors. MCCP tumors are characterized as having a low tumor mutational burden (TMB) while MCCN tumors typically have a high TMB with a UV-mutational signature^{50,51,52,53,54}. In particular, both Knepper et al. and Starrett et al. found that MCC tumors with a $TMB \geq 20$ mutations/MB were virus-negative while tumors with a $TMB \leq 6$ were virus positive^{53,54}. De-

spite the clear genetic differences, MCCP and MCCN tumors phenotypically present very similarly as described above. It is thought therefore that the activities of the viral proteins compensate for the lack of mutations and disrupt key driver pathways necessary for oncogenesis. In the next sections, we will discuss the known molecular pathways that are disrupted either by mutation or by viral protein activity in MCC.

1.2.2 MECHANISMS OF TUMOR SUPPRESSOR INHIBITION AND ONCOGENE ACTIVATION

pRB AND LT

As mentioned above, LT contains a pRB binding domain that contains the well-described pRB binding motif, the LXCXE motif. pRB (*RB1* gene) was the first tumor suppressor gene identified and is a key regulator of cell cycle progression, specifically regulating the transition from G1 to S (reviewed in⁵⁵). Once the cell progresses past G1 and into S, it is committed to replicating its DNA and ultimately undergoing cell division. pRB activity prevents cell cycle progression by binding to and inhibiting activating E2F proteins, transcription factors that promote expression of cell cycle genes. The *RB1* gene is mutated in about 5% of all cancers⁵⁶ and is functionally inactivated in many more^{57,58}.

In MCCN tumors, *RB1* is frequently mutated while in MCCP tumors *RB1* is typically wild-type: over 75% of TMB-high tumors reportedly had a *RB1* mutation while less than 10% of TMB-low tumors had a *RB1* mutation⁵³. Instead, in MCCP tumors LT binds to and functionally inactivates pRB, thereby relieving repression on activating E2F transcription factors and allowing for uninhibited cell cycle progression. Characterization of MCV integration sites in MCCP tumors has shown that the LT gene is typically mutated or truncated to remove the helicase domain but these mu-

tations invariably preserve the N-terminal side of the MCV early region proteins, including the J domain, full-length ST, and the pRB binding domain⁵⁹ (Figure 1.1C). This finding supports the understanding that inhibition of pRB by LT is crucial to MCC development.

L-MYC AND THE SLAP COMPLEX

In addition to *RB1* mutations, another characteristic of many neuroendocrine tumors is that they are poorly differentiated and MYC-driven^{60,53}. The MYC family of proto-oncogenes is a family of transcription factors that promote cell growth and division that when inappropriately activated can drive oncogenesis. This family includes c-MYC (the foundational member, often referred to as simply MYC), N-MYC, and L-MYC. In about 40% of MCCN tumors, the genomic region containing *MYCL1* is amplified^{61,54}. Recently, it was shown that MCV ST can directly bind to L-MYC and the TIP60/P400 complex, a large multifunctional chromatin remodeling complex, to form a ST, L-MYC, and P400 (SLaP) complex⁴⁸.

The TIP60/P400 complex has previously been implicated in promoting MYC-driven transcriptional programs⁶², so it seems that MCV ST increases inappropriate MYC target gene activation by facilitating an L-MYC-TIP60/P400 association. ChIP-seq and RNA-seq experiments performed with MCCP cell lines revealed that genes directly bound by the SLaP complex at the promoter had reduced expression following knockdown of P400, L-MYC, or ST/LT⁴⁸. Additionally, target gene promoters were enriched for E-box motifs, the canonical MYC binding motif, suggesting that a MYC regulatory network is activated by SLaP. These data suggest that the SLaP complex acts as a transcriptional activator, promoting the transcription of a subset of genes.

A few notable targets of the SLaP complex include LSD1, RCOR2, and INSM1,

components of the CoREST transcriptional repressor complex⁶³. Multiple groups have shown that this complex regulates expression of genes involved in neuroendocrine differentiation to favor a de-differentiated state in MCC^{63,64}. These findings further support the model that activities of the T antigens activate oncogenic programs which can phenotypically mimic the characteristics of non-viral neuroendocrine cancers.

P53

A notable difference between MCV LT and other polyomavirus LT proteins is that MCV LT does not have p53 binding activity^{65,66}. Dysregulated pRB inhibition is thought to stimulate p53 activity through induction of ARF (reviewed in⁵⁷), and indeed recent work has shown that expression of MCV LT leads to ARF expression and p53 activation⁶⁷. p53 (*TP53* gene) is another tumor suppressor transcription factor that when activated can trigger transcriptional programs to induce cell cycle arrest or apoptosis⁶⁸. Nearly 100% of MCCN tumors have a mutated *TP53* gene⁵³, suggesting that inhibiting the p53 pathway is critical for MCC development. However, the *TP53* gene is often wild-type in MCCP (about 90% of cases⁵³). It seems necessary therefore for both tumor development and viral replication that there is another virally-driven mechanism to functionally inactivate p53.

As there are very few other viral proteins expressed in MCC, it seems likely that p53 is inhibited indirectly by ST expression. Indeed, Park et al. found that expression of ST dampens the p53 response induced by LT by activating expression of MDM2 and CK1 α through the activities of the SLaP complex⁶⁷. MDM2 and CK1 α are both negative regulators of p53. MDM2 inhibits p53 by directly binding to prevent its transcriptional activity and to target it for ubiquitination (reviewed in⁵⁷. MDM4, or MDMX, is another p53 binding protein that can inhibit p53 transcriptional activity through di-

rect p53 binding. Additionally, MDM4 complexes with MDM2 to promote polyubiquitination of p53 by MDM2 (reviewed in⁶⁹). CK1 α is a kinase that stimulates MDM4-p53 binding by relieving MDM4 autoinhibition⁷⁰. Both MDM2 and MDM4-CK1 α can independently inhibit p53 transcriptional activity and together can promote p53 degradation through polyubiquitination.

Inhibition of MDM2, MDM4, and CK1 α is a therapeutic strategy actively being considered for cancers with functionally inactivated p53^{71,72,73}. In MCC, MDM2 inhibitors have been shown to be effective against *TP53* wild-type tumors^{67,73}, and an MDM2 inhibitor alone or in combination with avelumab is currently being investigated in a clinical trial (ClinicalTrials.gov Identifier: NCT03787602). These exciting findings highlight the potential for translating mechanistic studies examining the oncogenic targets of the SLaP complex into a clinical setting.

1.3 ARGININE METHYLATION AS A TARGETABLE FEATURE OF P53 WILD-TYPE TUMORS

As discussed above, the SLaP complex was shown to regulate the expression of genes essential to MCCP viability, including MDM2, CK1 α , LSD1, RCOR2, and INSM1^{67,63}. We asked whether there were any additional SLaP targets that represent therapeutic vulnerabilities for MCC and identified several protein arginine methyltransferase proteins, or PRMTs, as SLaP targets including PRMT1, 3, 5 and 7⁴⁸. PRMT5 in particular was of interest to us as a number of groups have found that PRMT5 inhibition can activate a p53 response through regulation of MDM4 in p53 wild-type cancers^{74,75,76,77}. Here, we will discuss the current understanding of arginine methylation and in particular the role of PRMT5 in oncogenesis.

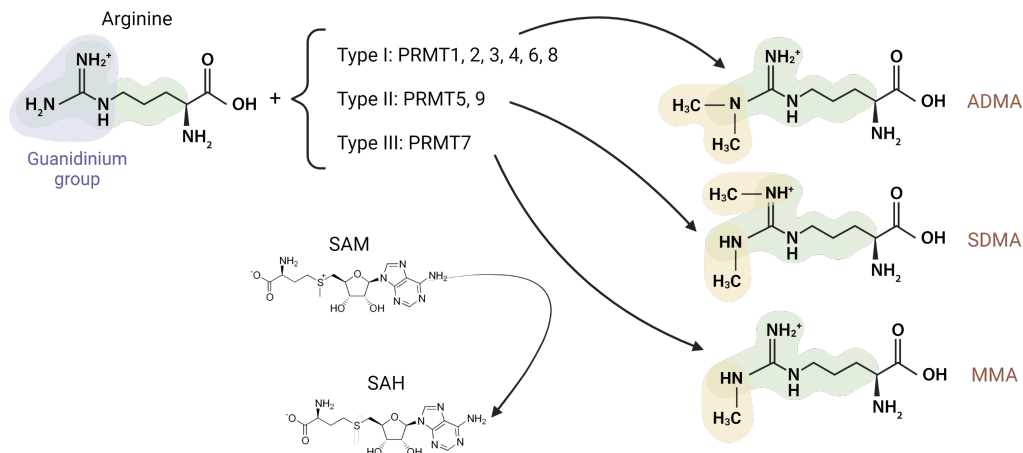


Figure 1.3: Arginine methylation is catalyzed by three types of PRMTs. Type I PRMTs catalyze the addition of asymmetric dimethylarginine (ADMA); type II PRMTs catalyze the addition of symmetric dimethylarginine (SDMA); type III PRMTs catalyze the addition of monomethylarginine (MMA). All PRMTs use S-adenosylmethionine as a methyl donor, ultimately producing methylarginine and S-adenosylhomocysteine (SAH). Created with BioRender.com.

1.3.1 PROTEIN ARGININE METHYLTRANSFERASES (PRMTs)

Arginine methylation is thought to play a role in many biological processes, including transcription, cell signaling, mRNA translation, DNA damage signaling, and pre-mRNA splicing and is required for important physiological functions during development, adult homeostasis, and aging (reviewed in ^{78,79,80}). Nine PRMT proteins have been identified in mammals, numbered from PRMT1 to 9. These proteins are further divided into type I, type II, or type III methyltransferases (Figure 1.3). Type I enzymes include PRMT1, 2, 3, 4, 6, and 8 and are responsible for catalyzing asymmetric dimethylarginine (ADMA) marks. Type II enzymes include PRMT5 and 9 and are responsible for symmetric dimethylarginine (SDMA). PRMT7 is the only member

of the type III family and catalyzes the formation of monomethylarginine (MMA). All PRMTs catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the terminal guanidino nitrogen atoms of arginine resulting in methyl-arginine and S-adenosylhomocysteine (SAH). Notably, PRMT5 forms a hetero-octomer complex composed of four subunits of PRMT5 and four subunits of WDR77 (MEP50) to form what is called the methylosome. The formation of this complex has been shown to be essential for PRMT5 methylation activity *in vitro*.

PRMTs typically target arginine- and glycine-rich motifs (RGG/RG motifs) to alter nucleic acid binding or protein-protein interactions. By introducing a methyl group, arginine methylation is thought to impact hydrogen bonding without changing the overall charge due to the steric effects of the methyl group. Arginine methylation increases interactions with Tudor domains however due to the relative sparsity of proteins with Tudor domains (36 human proteins) compared with the abundance of RGG/RG motifs (>2,000) it is thought that there exist additional methylarginine interaction domains that have yet to be identified.

Arginine demethylation is a poorly understood process with the identification of arginine demethylases being somewhat limited and controversial (reviewed in⁸¹). Jummanji C (JmjC)-domain-containing enzymes are a family of enzymes known to catalyze the removal of lysine methylation and are currently considered the most likely proteins responsible for arginine demethylation. A number of JmjC-domain-containing proteins have been shown to demethylate arginine residues *in vitro*. JMJD6 in particular, a JmjC-domain-containing protein, is a known lysine demethylase and was reported in multiple studies to remove arginine methylation marks *in vitro*. However, additional work found that JMJD6 was not able to demethylate arginine residues in known histone and non-histone targets and instead had lysyl-hydroxylase activity. Additional

work is necessary to determine whether any JmjC-domain-containing enzymes catalyze the removal of arginine methylation marks *in vivo*. Overall, arginine methylation is thought to be a relatively stable post-translational mark.

Of the PRMTs, PRMT5, PRMT1, and PRMT4 (also known as CARM1) are the most highly expressed in cancer, with high expression correlating with a worse prognosis. Cancer research efforts have led to clinical trials for inhibitors of PRMT5 and PRMT1, although the bulk of mechanistic studies and drug discovery efforts have been focused on PRMT5. In the next section, we will review the known targets of PRMT5 and our current understanding of how PRMT5 activity plays a role in important biological processes.

1.3.2 PRMT5 FUNCTION AND TARGETS

The mechanism of arginine methylation by PRMT5 specifically has been the focus of many studies over the years. PRMT5 forms a hetero-octamer complex with WDR77 (also known as WD45 or MEP50) to form what has been called the methylosome (reviewed in ⁷⁹). Additionally, PRMT5 activity can be modulated by three adaptor proteins that all bind to a specific region of PRMT5 in a mutually exclusive manner and these are CLNS1A (also known as pICln), RIOK1, and COPR5 (*COPRS* gene)^{82,83}. Each adaptor protein is thought to promote methylation of specific targets: CLNS1A recruits Sm and LSm proteins to regulate mRNA splicing and processing; RIOK1 recruits nucleolin and RPS10 to promote regulation of ribosome biogenesis; and COPR5 recruits nucleosomes to regulate chromatin remodeling and transcription. It's important to note however that there are many additional PRMT5 targets that have not been linked to any adaptor proteins⁸³. It is possible that there are additional adaptor proteins that haven't yet been identified or that PRMT5 methylates certain substrates

without any adaptor proteins. In this section, we will further discuss the PRMT5 targets that have been identified most consistently across cell lines and conditions, including proteins involved in regulating protein translation, mRNA splicing, transcription, and the DNA damage response.

PROTEIN TRANSLATION

Protein synthesis from translation of mRNA transcripts is a fundamental cellular process that has been shown to be regulated by PRMT5. One way in which PRMT5 activity influences translation is by regulating ribosome biogenesis. The ribosome is a complex molecular machine composed of numerous proteins and RNA molecules that is responsible for synthesizing proteins (reviewed in⁸⁴). The eukaryotic ribosome consists of two multi-protein and RNA-containing subunits known as the small 40S subunit and the large 60S subunit. Ribosome biogenesis occurs primarily in the nucleolus and is mediated by about 200 ribosome assembly factors and 80 small nucleolar RNAs (snoRNAs). PRMT5 with the adaptor protein RIOK1 has previously been shown to methylate both nucleolin, a nucleolar protein that regulates rRNA processing⁸⁵ and transcription of rRNA (reviewed in⁸⁶), and RPS10, a component of the 40S ribosomal subunit^{87,82}. Methylation of nucleolin is thought to modulate its interaction with nucleic acids⁸⁸ while methylation of RPS10 was shown to be required for ribosome assembly⁸⁷. PRMT5 regulation of ribosome biogenesis is likely pro-oncogenic as tumor cells generally have higher metabolic and protein synthesis demands.

There are a number of additional PRMT5 targets that influence protein translation including HNRNPA1 and SERBP1^{83,89,90}. HNRNPA1 is a member of the family of heterogeneous nuclear ribonucleoproteins that regulate many aspects of RNA metabolism⁹¹. HNRNPA1 in particular promotes IRES (internal ribosome entry site)

-dependent translation of genes including MYC and CCND1⁹² and methylation of HNRNPA1 by PRMT5 is required for this process⁹³. SERBP1 can reversibly repress translation by binding to and inhibiting ribosome function⁹⁴, however the role of arginine methylation in regulating SERBP1 activity is not well understood. Additional work is required to more clearly detail the many mechanisms of PRMT5 regulation of ribosome activity and to characterize how these processes contribute to oncogenesis.

THE SPLICEOSOME AND SPLICING FACTORS

A well-described function of PRMT5 is to regulate mRNA splicing. PRMT5 has been shown to directly methylate Sm proteins which are core components of the spliceosome^{95,96,97,98} as well as splicing factors such as SRSF1 and SFPQ⁸⁹. Methylation of Sm proteins is thought to be promoted by the adaptor protein CLNS1A and required for association with the SMN complex and proper spliceosome assembly (reviewed in⁷⁹). SRSF1 methylation was found to be required for AML cell line survival, suggesting that cell death due to PRMT5 inhibition is due at least in part to SRSF1 function. PRMT5 inhibition in these cells lead to differential splicing of over 1,000 exons and 400 retained introns, several which were in essential genes⁸⁹. Changes in alternative splicing as a result of PRMT5 inhibition have been reported in many other cancer cell lines as well^{99,76,100,101,74}. Although many additional targets of PRMT5 have been identified, it is important to consider both the direct effects of PRMT5-mediated methylation on cellular pathways and the indirect effects from protein level changes due to splicing defects.

HISTONE AND NON-HISTONE TRANSCRIPTIONAL REGULATORY PROTEINS

PRMT5 has been shown to target arginines on histones H3 and H4, and methylation of these targets by PRMT5 is promoted by COPR5¹⁰². The effect of this mark varies depending on the system and target gene studied. Methylation of H3R8 and H4R3 represses transcription of certain genes, such as RB1, RBL1, and RBL2 in leukemia and lymphoma^{103,104,105,106} but is reported to have an activating effect on others such as eIF4E and FGFR3 in colon and prostate cancers^{107,108}. Repression of RB1 and activation of eIF4E have been linked to oncogenesis but PRMT5-mediated histone methylation has also been linked to repression of c-MYC in pancreatic islet tumor cells which has a tumor suppressive effect¹⁰⁹. Similarly, PRMT5 has been shown to methylate transcriptional regulators such as P53^{110,111} and E2F1¹¹² which have tumor suppressive and oncogenic effects respectively.

DNA DAMAGE RESPONSE PROTEINS

The p53 pathway is a critical DNA damage response (DDR) pathway that can react to diverse DNA damage stimuli to activate expression of DDR proteins, arrest the cell cycle, or induce apoptosis (reviewed in¹¹³). PRMT5 activity has been implicated in regulating the p53 response through both direct methylation and splicing regulation. P53 has been hypothesized to sense splicing defects through alternative splicing of the MDM4 transcript, either through exon skipping^{74,75,77} or through intron retention¹¹⁴. Alternative splicing of MDM4 produces either an unstable protein or a decrease in transcript levels, leading to reduced total levels of MDM4 and allowing for p53 activation. Loss of PRMT5 activity was previously shown to promote alternative splicing of MDM4, and *TP53* status was found to correlate with PRMT5 inhibitor sensitivity

across a broad range of cancer cell lines⁷⁵. P53 has also been identified as a direct target of PRMT5, and it has been suggested that loss of p53 arginine methylation alters the promoter binding specificity of p53 leading to a pro-apoptotic response¹¹⁰.

In addition to direct and indirect regulation of the p53 response, PRMT5 has been implicated in regulating DDR proteins upstream of p53 through both direct methylation and splicing defects. There are many different branches of the DDR including nucleotide excision repair, mismatch repair, and double strand break (DSB) repair¹¹⁵. DSBs are typically thought of as the most toxic form of DNA damage, and much of the current literature on the role of PRMT5 in regulating the DDR centers on the DSB repair response. For example, previous studies have shown that PRMT5 loss leads to a defect in DSB repair by the homologous recombination (HR) response, a pathway that resolves DSBs with high fidelity by using a homologous DNA strand as a template sequence for repair. One proposed mechanism of HR regulation by PRMT5 is that PRMT5 methylates RUVBL1 which is required for recruitment of TIP60 to DSBs, a step critical for ATM activation and DSB repair by HR¹¹⁶. Hamard et al. however found no evidence for RUVBL1 methylation and instead proposed a slightly different model. They reported that loss of PRMT5 leads to improper TIP60 splicing which reduces expression of the canonical TIP60 isoform, TIP60 α which in turn reduces ATM activation and hinders DSB repair by HR⁷⁶. Despite some mechanistic discrepancies, both studies argue that PRMT5 loss induces a defect in the DSB response which can lead to cell death due to an accumulation of unrepaired DSBs as well as heightened vulnerability of the cell to DNA damaging agents.

1.4 UNDERSTANDING MOLECULAR MECHANISMS TO IDENTIFY NOVEL TARGETED THERAPIES IN MERKEL CELL CARCINOMA.

In this dissertation, we investigate the molecular mechanisms underpinning Merkel cell carcinoma biology to identify PRMT5 as a promising therapeutic target. In Chapter 2, we investigate the role of TIP60 in SLaP complex gene regulation and find that acetylation of histone H2A.Z is disrupted by both TIP60 knockdown and TIP60 overexpression. We show that H2A.Z and H2A.Z acetylation correlate with SLaP complex binding genome-wide, supporting a model that TIP60/P400 complex activates SLaP target genes by promoting an active chromatin state.

In Chapter 3, we explore the therapeutic potential of PRMT5, a downstream target of the SLaP complex. We show that most MCC cell lines are sensitive to pharmacological inhibition of PRMT5 and treatment with PRMT5 inhibitors induces cell cycle arrest and apoptosis. We show that PRMT5 inhibition induces a p53 response but find that PRMT5 inhibitor-mediated cell cycle arrest and apoptosis is predominantly p53 independent.

In Chapter 4, we identify mRNA processing as a key pathway regulating PRMT5 inhibitor sensitivity. We investigate the effect of PRMT5 loss on mRNA splicing in MCC and find that PRMT5 inhibition significantly increases levels of detained introns in DNA damage and cell cycle genes. We further characterize the effect of PRMT5 on the proteome and find that PRMT5 loss increases expression of proteins involved in mRNA processing. We test the assumption that splicing changes directly alter protein expression and find that changes on the RNA level due to PRMT5 inhibition only weakly correlate with protein level changes. We show that taking a splice-aware approach to calculate the proportion of productive transcripts improves this correlation

for only a subset of genes, although the correlation remains quite low.

In Chapter 5, we investigate the role of PRMT5 in regulating the DNA damage response. We find that PRMT5 inhibitors cause more DNA damage in PRMT5i-sensitive than PRMT5i-resistant MCC cell lines, suggesting that DNA damage accumulation may drive PRMT5i sensitivity. We show that this DNA damage signal appears earliest in S phase cells, suggesting that PRMT5 inhibition increases levels of replication stress. We further characterize the effect of PRMT5 inhibition on the DNA damage response and find that PRMT5 loss leads to a defect in ATM expression and causes cells to preferentially repair DSBs via non-homologous end joining. We show that PRMT5 inhibition causes a defect in ATR signaling in response to irradiation or hydroxyurea treatment leading to an increase in DNA-PKcs activation. Taken together, we propose a model where splicing dysregulation due to PRMT5 inhibition increases replication-associated DNA damage while simultaneously reducing the ability of the cell to respond to replication stress by inhibiting both ATM activity and the ATR/CHK1 pathway.

AUTHOR CONTRIBUTIONS FOR CHAPTER 2

The experiments in this chapter were performed by Camille H. Cushman with input from James A. DeCaprio. This work was supported in part by the US Public Health Service grants F31CA239345 to C.H.C. and R35CA232128 and P01CA203655 to J.A.D.

2

Characterizing the role of TIP60 acetyltransferase activity in gene transactivation by the SLaP complex.

2.1 ABSTRACT

Investigating the molecular activities of polyomavirus tumor antigens has previously led to advancements in our understanding of cancer development both in the context

of viral oncogenesis and in non-viral cancers. Merkel cell polyomavirus (MCV) is a human polyomavirus known to cause an aggressive neuroendocrine skin cancer known as Merkel cell carcinoma in the majority of cases. Recent work has identified a transcriptional regulatory complex composed of the MCV small tumor antigen (ST) bound to L-MYC and the TIP60/P400 complex. This complex, referred to here as the SLaP complex, was shown to activate an oncogenic transcriptional program necessary for MCC growth and survival. In this chapter, we examined the expression and activities of the histone acetyltransferase TIP60 in an effort to better characterize the mechanism of SLaP-mediated gene activation. We showed that multiple TIP60 isoforms are expressed in MCC and that they all can co-precipitate with the SLaP complex. Using shRNA knockdown of TIP60, we found that TIP60 loss leads to a loss of MCC cell viability and a decrease in both H4K16 and H2A.Z histone acetylation. However, expression of shRNA-resistant TIP60 cDNA did not rescue the cell viability or histone acetylation defects we observed, suggesting off-target effects. Interestingly, overexpression of TIP60 led to a decrease in total H2A.Z levels indicating that high levels of TIP60 negatively regulate H2A.Z expression. We examine H2A.Z and H2A.Z acetylation levels genome-wide and find that both are enriched at promoters bound by the SLaP complex. We conclude that H2A.Z acetylation may contribute to activation of SLaP target genes, but additional methods are necessary to carefully evaluate the role of TIP60 isoforms in both gene regulation by the SLaP complex and H2A.Z expression.

2.2 INTRODUCTION

Identification of the ST, L-MYC, and P400 complex (SLaP complex) revealed key insights into the biology of MCCP⁴⁸, in particular identifying mechanisms of functional p53 inactivation through activation of MDM2 and CK1 α expression⁶⁷ and of maintaining a de-differentiated state through activation of LSD1, RCOR2, and INSM1 expression⁶³. Based on the work by Cheng et al.⁴⁸, we expect that ST promotes activation of L-MYC target genes by recruiting the TIP60/P400 chromatin modifying complex to the target gene promoters. Not only are SLaP binding sites enriched for E-box motifs, the canonical MYC binding motif, but shRNA-mediated knockdown of P400 or L-MYC reduced expression of the genes directly bound by SLaP⁴⁸, suggesting that this complex acts as a transcriptional activator. Given that neither ST nor L-MYC/MAX contain any catalytic activity, it seems likely that the catalytic activities of the TIP60/P400 complex play a direct role in promoting gene transactivation at SLaP target genes.

The TIP60/P400 complex is a large, multi-subunit complex that contains both lysine acetyltransferase and chromatin remodeling activity. TIP60 (*KAT5* gene) reportedly acetylates a number of histone and non-histone targets and has been described as a hub protein, interacting with multiple complex regulatory pathways (reviewed in^{117,118}). Although TIP60 forms multiple complexes, it is generally thought that the majority of TIP60 exists in the TIP60/P400 complex¹¹⁹. In MCC specifically, gel-filtration experiments showed that the majority of TIP60 was contained in the SLaP complex⁴⁸.

An underappreciated feature of TIP60 biology is that the *KAT5* gene is alternatively spliced to form at least 4 distinct TIP60 isoforms. The sequences of these iso-

forms can be accessed on both the NCBI and UniProt databases, however the isoforms are numbered differently between databases. These isoforms are generated by the alternative splicing or inclusion of two regions of the KAT5 gene. The best studied TIP60 isoform by far is TIP60 α (also known as isoform 2, NCBI and UniProt) which is considered the canonical protein sequence. TIP60 α is generated by splicing out the first region, hereafter referred to as intron 1, and by including the second region, also known as exon 5. Intron 1 is a 33-amino acid sequence that is in frame with the canonical coding sequence while exon 5 contains 52 amino acids. TIP60 β is the next best studied isoform (also known as isoform 3 [NCBI] or isoform 1 [UniProt]) which is generated by splicing out both intron 1 and exon 5. There is very little known about the last two isoforms which differ from α and β by the inclusion of intron 1. Here, we will refer to these isoforms as TIP60 α +i and TIP60 β +i. All TIP60 isoforms contain a chromodomain, a zinc finger domain, and an acetyl CoA binding domain. The chromodomain is important for recognition of methylated histone marks, specifically H3K9me3, while the zinc finger domain is required for H4 tail recognition and the acetyl CoA binding domain is required for the catalytic activity of TIP60 (reviewed in ^{118,120}). Despite the similarity between TIP60 α and TIP60 β sequences, differences in their cellular localization patterns suggests they may have distinct activities. Ran et al. found that TIP60 α localizes primarily to the nucleus in HeLa cells while TIP60 β localizes to both the nucleus and the cytoplasm ¹²¹.

The histone targets of TIP60 α are reported to include histone H4 at lysines K5, K8, K12, and K16 (reviewed in ¹¹⁷). More recently, TIP60 α has been linked to acetylation of the H2A histone variant, H2A.Z. In *Saccharomyces cerevisiae* and *Drosophila*, the TIP60 homolog is responsible for acetylation of H2A.Z (reviewed in ¹²²) and there are reports suggesting this is also the case in mammalian cells ^{123,124}. Histone acetylation

has long been linked to transcriptional activation via chromatin relaxation, particularly histone H4K16ac, although H4ac in general is considered a mark of active transcription (reviewed in ¹²⁰).

TIP60 β reportedly forms a very similar or possibly identical TIP60/P400 complex as what has been described for TIP60 α . Doyon et al. reported that immunoprecipitation of tagged TIP60 α and TIP60 β produced indistinguishable protein complexes, and purified TIP60 β complexes had greater HAT activity on oligonucleosomes than purified TIP60 α complexes ¹¹⁹. In contrast, Hamard et al. reported that purified TIP60 α complexes have approximately 2-fold greater HAT activity towards H4 and H2A and equivalent activity towards H2AX as purified TIP60 β constructs ⁷⁶. It is possible that distinct TIP60-containing complexes have unique acetylation targets, play unique roles in regulating gene transcription, and/or have cell type-specific functions.

Another member of the TIP60/P400 complex with catalytic activity is P400 (*EP400*). P400 is an ATPase in the INO80 family of chromatin remodelers which are characterized by a split ATPase domain and the association with two RuvB-like proteins, RUVBL1 and 2 (reviewed in ¹²⁵). In mammalian cells, P400 is thought to be responsible for H2A.Z deposition ^{126,127} and has been shown to exchange canonical nucleosomes for H3.3/H2A.Z-containing nucleosomes in vitro ¹²⁸. Although H2A.Z is negatively correlated with gene expression, H2A.Z in the promoter is required for optimal activation of certain genes ^{129,130}, and acetylated H2A.Z is positively correlated with gene expression ¹³¹. Acetylated H3.3/H2A.Z-containing nucleosomes are associated with active transcription and stimulate transcriptional activity in vitro ¹²⁸. These findings suggest that the TIP60/P400 complex can stimulate gene expression through both histone acetylation and histone variant deposition.

Although data in MCC suggested that the interaction between L-MYC/MAX and the TIP60/P400 complex is dependent on ST⁴⁸, the interaction between c-MYC/MAX and the TIP60/P400 complex has been well-documented, especially in the context of development^{132,133,134,135}. The TIP60/P400 complex was found to be present at 50-60% of MYC binding sites, most active promoters, and even at a subset of well-defined enhancers in mouse embryonic stem cells (mESCs)¹³⁵. Loss of TIP60 or TRRAP, a scaffold subunit of the complex, is embryonic lethal^{136,137} and results in cell cycle defects in tissue culture¹³⁸. However, the TIP60/P400 complex has also been linked to gene repression. Although Fazzio et al. report bulk H4ac loss following TIP60 knockdown in ESCs, they report that the effect of TIP60 or P400 knockdown on gene expression is primarily gene activation and ultimately conclude that the TIP60/P400 complex is required for transcriptional repression in ESCs¹³⁸. Additional groups have identified repressive roles for both TIP60 and P400^{139,140} so further work is necessary to fully understand the relationship between TIP60/P400 complex activity and gene expression, especially keeping in mind that there may exist multiple distinct TIP60/P400 complexes containing unique TIP60 isoforms.

We hypothesized that the acetyltransferase activity of TIP60 would be required for oncogenic gene transactivation by the SLaP complex. We evaluated TIP60 isoform expression and discovered that all four TIP60 isoforms are expressed and can co-precipitate with components of the SLaP complex in MCC. ShRNA-mediated knockdown of TIP60 was lethal to MCC cells and resulted in decreased levels of H2A.Z acetylation, however these effects could not be rescued with overexpression of the major TIP60 isoforms, TIP60 α and TIP60 β . In fact, overexpression of both major isoforms actually reduced total levels of H2A.Z and H4K16ac. To look for evidence of TIP60 and P400 catalytic activity, we focused on the distribution of acetylated H2A.Z

marks throughout the MCC genome and found that H2A.Z acetylation overlapped with SLaP binding sites at gene promoters by ChIP-seq. We conclude that although there is some supporting evidence of TIP60/P400 activity at SLaP target genes, additional strategies to target specific TIP60 isoforms are necessary to more clearly delineate the role of TIP60 acetyltransferase activity in gene activation mediated by the TIP60/P400 complex.

2.3 METHODS

2.3.1 CELL CULTURE

MCC cell lines were gifts from Masahiro Shuda (University of Pittsburgh), Jürgen Becker (Translational Skin Cancer Research, German Cancer Consortium (DKTK), Essen, Germany), and Roland Houben (University of Wuerzburg, Germany). Cells were cultured in RPMI (Life Technologies, 11875119) with 10% FBS and 1% penicillin-streptomycin (Life Technologies 15140163). Cell lines were confirmed to be free of mycoplasma contamination using the e-Myco Mycoplasma PCR Detection Kit (Bulldog Bio 25233).

2.3.2 RT-PCR OF TIP60 ISOFORMS

Total RNA was purified from MKL-1 cells using the RNeasy Plus Mini kit (Qiagen 74034). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher 4368814) with random primers according to the manufacturer's instructions. The cDNA was purified using the QIAquick PCR purification kit (Qiagen 28104) and quantified by nanodrop. The cDNA was then amplified using the Platinum SuperFi DNA polymerase (Invitrogen 12351010) according to the manu-

facturer's instructions and separated in a 1% agarose gel. The primers used to amplify the TIP60 isoforms from cDNA are listed in Table 2.1:

Table 2.1: TIP60 RT-PCR primers

Name	Sequence
Set 1 Forward	GGTGAATTCATGGCGGAGGTGGGGGAGAT
Set 2 Forward	GGTGAATTCATGGCGGAGGTGGTGAGTCCG
Reverse	GGTGTCTAGATCACCACTTCCCCCTCTTGCTC

2.3.3 IMMUNOPRECIPITATION AND IMMUNOBLOT

Cell pellets were lysed in EBC buffer and protein content was quantified by Bradford assay. For histone acid extraction, the Abcam histone extraction kit buffers (ab113476) were used according to the manufacturer's instructions instead of EBC. For immunoprecipitation, 500 ug - 1 mg of protein was incubated with antibodies according to the manufacturer's instructions (for Ab5, a dilution of 1:1000 was used) and protein A/G beads overnight. Beads were washed in EBC buffer and protein was eluted in 1X Laemmli sample buffer at 95C for 5 min. For immunoblot, lysates were separated on either a 4-20% or 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked in 5% milk, and incubated with primary antibody at 4°C overnight. A list of the primary antibodies used can be found in Table 2.2. Blots were imaged with either SuperSignal West Pico (ThermoFisher PI34080) or Immobilon Western Chemiluminescent HRP Substrate (Millipore WBKLS0500).

2.3.4 LENTIVIRUS INFECTION

Lentivirus was produced by transfecting 293T cells with a pLKO.1 plasmid containing the shRNA of interest (shRNA target sequences listed in Table 2.3) and lentiviral

Table 2.2: Antibodies used for immunoprecipitation or immunoblot

Target	Catalog Number
DMAP1	Santa Cruz SC-373949
H2A.Z	Abcam ab4174
H2A.Zac	Abcam ab232908
H3.3	Abcam ab176840
H4K8ac	CST 2594S
H4K12ac	CST 2591S
H4K16ac	CST 13534S
H4	CST 2935S
IgG rabbit	CST 2729S
IgG mouse (α -HA)	CST 2367
MAX	Bethyl A302-866A
P400 (EP400)	Bethyl A300-541A
pan-MYC	Abcam ab195207
ST	Ab5: Cheng et al. 2013 ⁶⁵ , 2017 ⁴⁸
TBP	CST 8515S
TIP60	Santa Cruz SC-166323
VINC	Sigma Aldrich V9131

second generation packaging vectors (psPAX2 and VSV-G) using polyethylenimine (PEI) in serum-free media. Lentivirus-containing media was collected 48 hours after transfection and filtered through a 0.45 μ m filter prior to use. For suspension cells (MKL-1, WaGa): Cells were spininfected as described in the Broad Optimization of lentiviral transduction using spinfection protocol (https://portals.broadinstitute.org/gpp/public/dir/download?dirpath=protocols/production&filename=Optimization_of_Lentiviral_Spinfection_Oct2018.pdf). For adherent cells (MCC13): Lentivirus was directly applied dropwise to cells and incubated at 37°C for 4-24 hrs.

Table 2.3: shRNA sequences

shRNA ID	Target sequence	Origin
TIP60-1	cctcaatctcatcaactacta	TRCN0000293404
TIP60-2	ccaggacggaagcgaaaatcga	Simpson et al. 2018 ¹⁴¹
TIP60-3	gctcccacaaagcactctaag	Designed in-house
TIP60-4	cagagggtgtgattgtaaa	Designed in-house
TIP60-5	cggcaataaattgtttctata	Designed in-house
TIP60-6	caagtgtcttcagcgtcattt	TRCN0000293417
TIP60-7	ggacgtaagaacaagagttat	TRCN0000293476
TIP60-8	tcgaattgtttgggcactgat	TRCN0000020318
TIP60-9	cctcctatcctatcgaagcta	Zhao et al. 2018 ⁶² ; TRCN000020317
EP400-1	gcaaagacatccacatataca	Cheng et al. 2017 ⁴⁸
EP400-2	tccgtacgagccagatctatg	TRCN0000370637
MYCL-1	tggcgcttagagaggacaata	TRCN0000415108
Ctrl	tcctaaggttaagtcgcctcg	Cheng et al. 2017 ⁴⁸

2.3.5 CELL VIABILITY ASSAYS

Cells were infected with lentivirus as described above and cultured for 2 days prior to selection in puromycin-containing growth media at a cell line-dependent concentration. After 2-3 days of selection, cells were collected, resuspended in 2 mL of growth media, and 20 uL was aliquotted into 96-well plates in triplicate with 80 uL growth media for cell viability measurements. The remainder of cells were re-plated in selection media. Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega G7571) according to the manufacturer's instructions with the following modifications: 25 uL of CellTiter-Glo® reagent was added per 100 uL sample and was incubated at RT on a shaker for 12 min prior to quantification.

2.3.6 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation was performed using the SimpleChIP® kit with a few modifications. Briefly, cells were fixed in 2mM disuccinimidyl glutarate (DSG)

and then fixed in 1% formaldehyde. Samples were washed in PBS and incubated in the SimpleChIP® Buffer A for 10 min at 4°C followed by another incubation step in Buffer B for 10 min at 4°C. Chromatin was enzymatically digested by MNase treatment in Buffer B at 37°C for 1 hr. The digest was stopped with 0.05M EDTA and samples were put on ice before being pelleted and resuspended in a buffer (referred to as Buffer 3) containing 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1 % Na-deoxycholate, and 0.5 % N-Lauroyl-sarcosine with protease inhibitors added fresh. Samples were then sonicated 3x, 20 sec on and 30 sec on ice, before additional N-lauroyl sarcosine was added to a final concentration of 1%. Samples were incubated at RT on a rocker for 10 min before pelleted and the supernatants were transferred to fresh tubes.

An aliquot of each sample was then taken for chromatin quantification and quality control assessment. The aliquots were treated with proteinase K for 4-24 hrs at 65°C before DNA was purified using the QIAQuick PCR purification kit, quantified by nanodrop, and separated on a 1% agarose gel to visualize fragmentation efficacy. Based on the concentrations of the aliquotted chromatin, 15 ug of the remaining sample were combined with equal parts Buffer 3 and RIPA buffer (50 mM HEPES-KOH pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP40, 0.7% Na-deoxycholate). An experimentally-determined amount of antibody and ChIP-grade protein G magnetic beads were added to each sample and incubated at 4°C overnight on rotation. A magnetic rack was then used to collect and wash beads in RIPA buffer 3x, followed by 1x wash in TE + 50 mM NaCl. Protein-DNA complexes were eluted from the beads in T₅₀E₁₀S₁ buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 15-30 min. Eluent was transferred to a fresh tube and the elution step was repeated using T₅₀E₁₀ buffer. Proteins were digested using proteinase K and DNA was purified using

the QIAquick PCR purification kit. Samples were then analyzed either by qPCR or by high-throughput sequencing.

HIGH THROUGHPUT SEQUENCING AND ANALYSIS

After DNA purification, library preparation and high-throughput sequencing was performed by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. Samples were sequenced single-end, 75bp with an Illumina Next-Seq 500. Data was analyzed using the ENCODE3 histone replicated ChIP-seq processing pipeline to call narrow peaks. Heatmaps were generated using the deepTools suite of python tools¹⁴².

2.4 RESULTS

2.4.1 MULTIPLE TIP60 ISOFORMS ARE INVOLVED IN SLAP COMPLEX ACTIVITY.

To understand how the SLAP complex promotes transcription, we decided to focus on the activities of the TIP60/P400 complex. TIP60 in particular can directly acetylate nucleosomes at lysines on histones H2A, H2A.Z, and H4 (reviewed in¹¹⁷). Histone acetylation has been widely accepted as a mark of active transcription (reviewed in¹²⁰), so we hypothesized that TIP60 acetyltransferase activity is required for SLAP target gene activation. However, multiple isoforms of TIP60 have been previously reported, and more than one has been shown to have acetyltransferase activity^{119,121,76}. In humans, there are 4 previously reported TIP60 isoforms that differ by the alternative splicing of either the first intron (intron 1) or the fifth exon (exon 5) (Figure 2.1A). The canonical sequence is TIP60 α while the second best-described isoform is TIP60 β and we will refer to these as the major isoforms. Both major isoforms exclude intron 1 but TIP60 α includes exon 5 while TIP60 β excludes this exon. The minor iso-

forms differ from the major isoforms by the inclusion of intron 1 and will therefore be referred to here as TIP60 α +i and TIP60 β +i. The NCBI isoform ID numbers are indicated to the left of the cartoon in Figure 2.1A for clarity. These isoforms all contain the chromodomain, zinc finger domain, and the acetyl co-A binding domain, so it is possible that they all have acetyltransferase activity.

To determine whether all TIP60 isoforms were expressed in MCC cells, we first performed RT-PCR of total RNA isolated from MKL-1 cells using primers specific to either the major isoforms or minor isoforms (Figure 2.1B). We found that all four isoforms were transcribed in MKL-1 cells, although the major isoforms were more readily detectable than the minor isoforms in an agarose gel. TIP60 β and TIP60 β +i transcripts appeared to be more abundant than their TIP60 α counterparts.

After confirming that all 4 TIP60 isoforms are expressed on the RNA level, we asked whether they were detectable on the protein level and if so, which isoforms could co-precipitate with the SLaP complex (Figure 2.1C). We performed immunoprecipitations (IPs) of MAX, P400, and ST (using Antibody 5, or Ab5, that recognizes the J domain of both ST and LT^{65,48}) and blotted for TIP60. We specifically used a TIP60 antibody that targeted the C terminal side of TIP60 in order to be able to detect all 4 TIP60 isoforms. By separating the lysate on a 7.5% gel, we were able to visualize all 4 TIP60 isoforms in our input fraction. Additionally, we could see that all 4 isoforms co-precipitated with MAX, P400, and ST. Much, but not all, of the TIP60 protein in the cell appears to be bound in the SLaP complex as seen by the depletion of TIP60 in the IP flow-through compared with the IgG flow through. These findings suggest that there are multiple SLaP complexes composed of unique TIP60 isoforms. Whether these complexes have interchangeable or unique functions is unknown.

To determine whether the acetyltransferase activity of TIP60 plays a crucial role

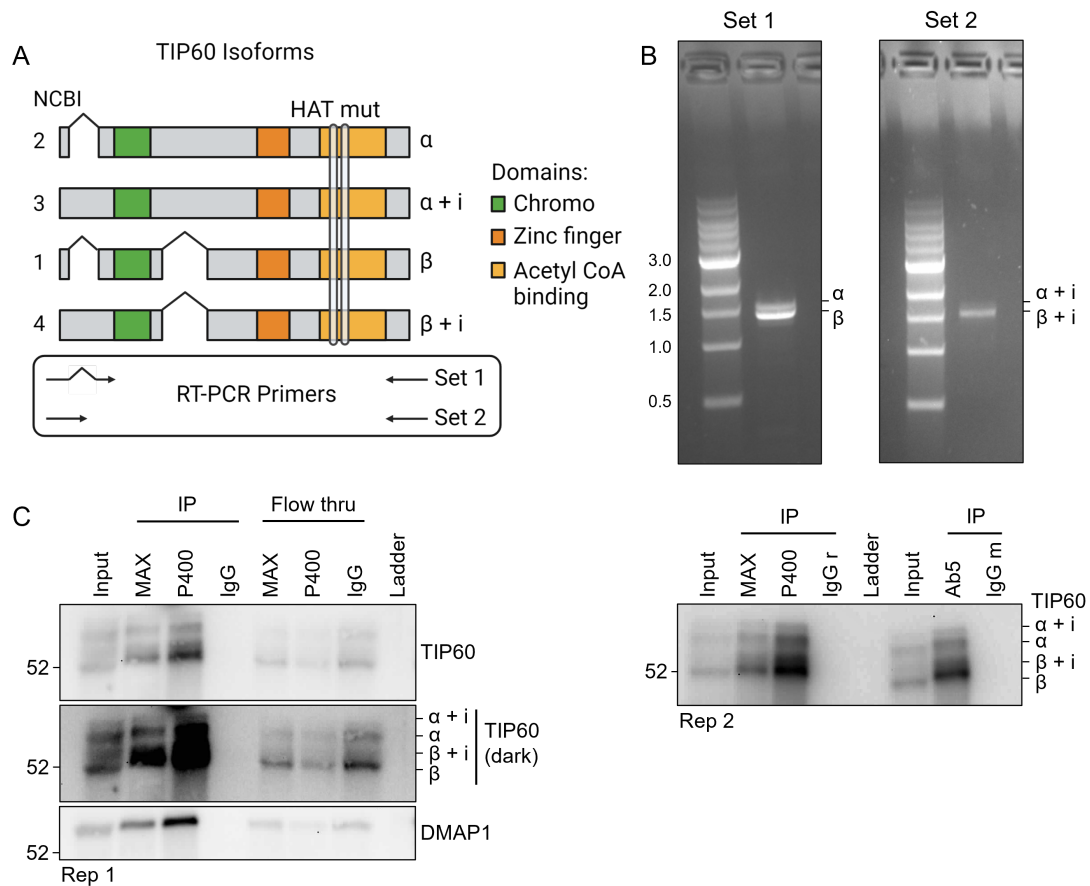


Figure 2.1: Four TIP60 isoforms are expressed in MCC cells and co-precipitate with the SLaP complex. **A.** Cartoon of previously annotated TIP60 isoforms including the NCBI transcript ID, the previously annotated protein domains, and the RT-PCR primer target sequences used to distinguish the major TIP60 α and TIP60 β isoforms from the minor TIP60 α +i and TIP60 β +i isoforms. The location of the two point mutations, Q377E and G380E, that inhibit TIP60 catalytic activity are highlighted. Created with BioRender. **B.** RT-PCR of major (left) and minor (right) TIP60 isoforms from MKL-1 cells. **C.** Immunoprecipitation of SLaP complex components with immunoblotting for TIP60 isoforms.

in the SLAP complex, we first evaluated the effect of TIP60 loss on MCCC viability. A CRISPR screen performed by Cheng et al. identified multiple components of the SLAP complex as required for MKL-1 cell viability including RUVBL1, RUVBL2, P400, and L-MYC⁴⁸. TIP60 was also identified in this CRISPR screen as essential but with a higher false discovery rate (>25%). To validate these findings, we evaluated a panel of shRNA against TIP60 (target sequences listed in Table 2.3) and identified two that successfully reduced TIP60 protein levels (Figure 2.2A). The target sequences of these shRNA are shared amongst all TIP60 isoforms, and indeed we see depletion of all by immunoblot. We then used these shRNA to knock down TIP60 in two MCCC cell lines (MKL-1 and WaGa) and one MCCN cell line (MCC13) and measured the effect of TIP60 loss on cell viability via CellTiter-Glo® (Figure 2.2B-E). We compared the effect of TIP60 loss to the effect of P400 and L-MYC loss and found that TIP60 knockdown reduced MCCC cell viability to a similar degree in both cell lines (Figure 2.2C). We evaluated the efficacy of the shRNA by immunoblot and saw a near complete loss of all TIP60 isoforms 7 days after shRNA expression (Figure 2.2D-E).

TIP60 α is considered the canonical TIP60 isoform, and most of the literature focuses on the activities of this isoform. It is unknown whether the other TIP60 isoforms have the same activities and play redundant roles or whether they have unique targets and functions in the cell. To parse out the different contributions of the isoforms to TIP60 function and cell viability, we asked whether expression of a single isoform, either TIP60 α or TIP60 β was sufficient to rescue TIP60 loss. We generated MKL-1 cells expressing shRNA-resistant TIP60 constructs, one wild-type (WT α and WT β) and one catalytically dead (mut α and mut β) for each shRNA. We first validated our system by immunoblot (Figure 2.3A-B) before testing whether these cells were resistant to the toxic effects of the TIP60 shRNA. We saw that neither TIP60 α

nor TIP60 β expression alone was sufficient to rescue cell viability (Figure 2.5C-D) in this system.

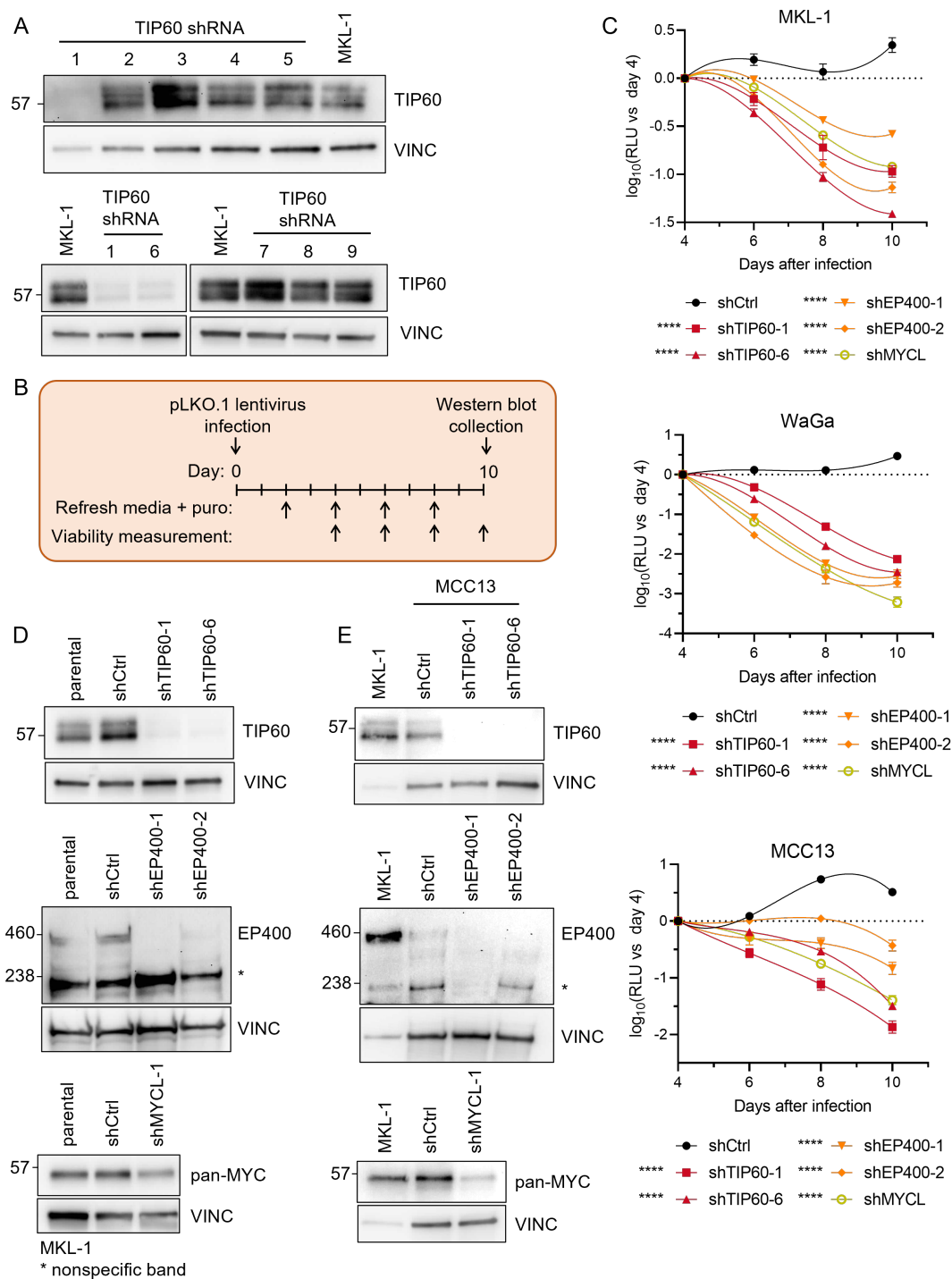
Although TIP60 α was not sufficient to rescue cell viability, we wanted to know whether TIP60 α could rescue acetylation defects as a result of TIP60 loss. TIP60 is widely reported to contribute to H4 acetylation, specifically on lysines K5, K8, K12, and K16. Another potential target of TIP60 is H2A.Z, a histone mark associated with both TIP60 acetyltransferase activity and P400 chromatin remodeling activity. To address this question, we knocked down TIP60 in our TIP60 α rescue lines or a GFP control line and collected cells 5 days later for immunoblot of both total and histone acid extracted protein (Figure 2.4). TIP60 knockdown with shTIP60-1 in GFP lines reduced H2A.Zac and H4K16ac levels without affecting total H2A.Z or H4 levels, suggesting that TIP60 is required for H2A.Z and H4K16 acetylation as previously reported.

We also blotted for histone H3.3, a histone H3 variant associated with P400 chromatin remodeling activity, and saw that TIP60 loss had no effect on total H3.3 expression. However, overexpression of shRNA-resistant TIP60 α did not provide any rescue of the H2A.Zac or H4K16ac loss. In fact, cells overexpressing TIP60 α appeared to have slightly lower levels of H2A.Z at baseline without impacting histone H4 levels. We performed a similar experiment using both TIP60 shRNA and found that shTIP60-6 affected total H2A.Z levels regardless of TIP60 rescue expression. Given that we don't see this effect using shTIP60-1, this data suggests that shTIP60-6 may have off-target effects.

Previous reports have suggested that both TIP60 α and TIP60 β have acetyltransferase activity^{119,121,76}, so to rule out the possibility that both TIP60 isoforms are essential in MCC we generated MKL-1 cells that express both shRNA-resistant TIP60 α

Figure 2.2 (following page): Expression of SLaP complex components are required for MCC cell viability. **A.** A panel of 9 different TIP60-specific shRNA were tested in MKL-1 cells by immunoblot. **B.** Experimental design of TIP60 shRNA knockdown experiments in C-E. **C.** Cell viability after shRNA expression via lentivirus infection was measured by CellTiter-Glo® assay and normalized to day 4. Statistics were calculated by two-way ANOVA compared to shCtrl. **** = $p < 0.0001$. Error bars represent SEM. N=3. **D-E.** Immunoblots of shRNA used in B. in MKL-1 cells (**D.**) and MCC13 cells (**E.**).

Figure 2.2: (continued)



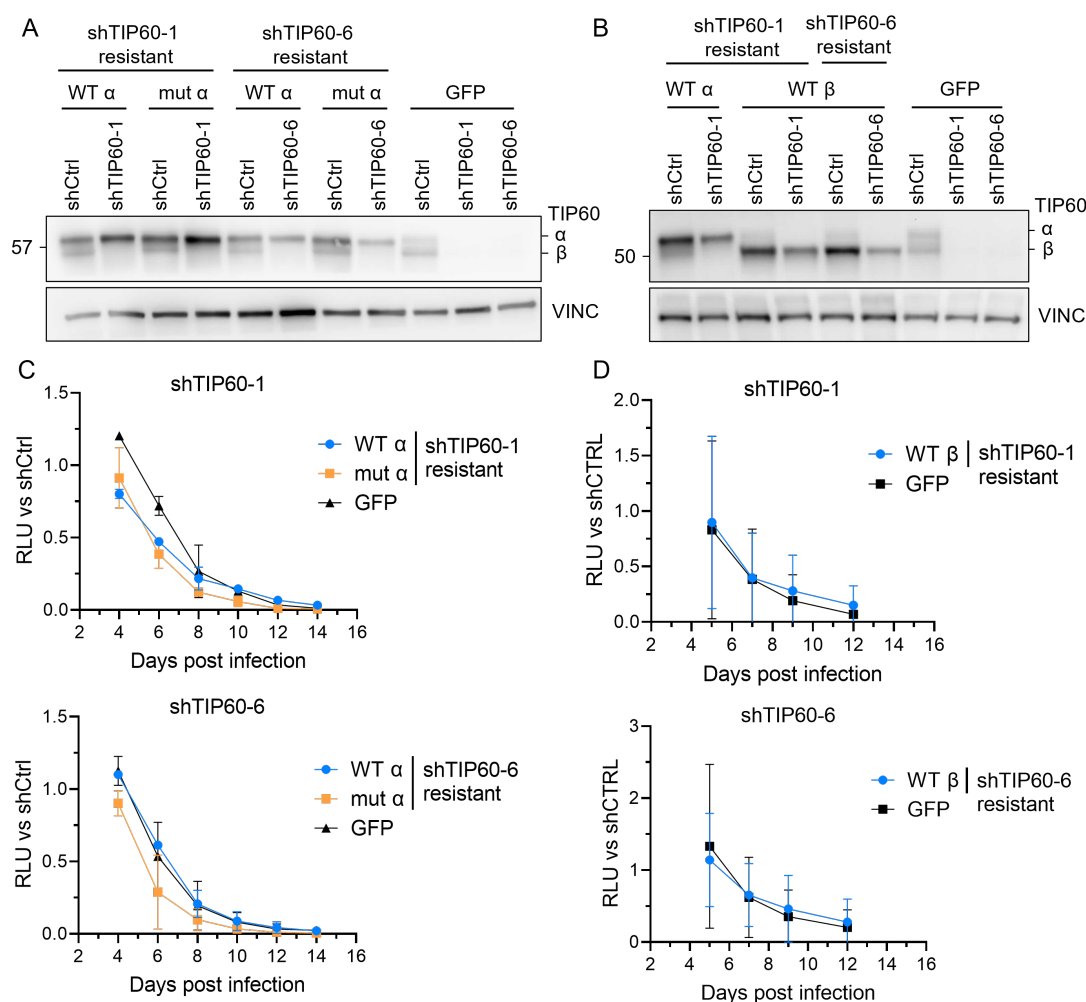


Figure 2.3: Overexpression of TIP60 α or β is not sufficient to rescue cell viability following TIP60 knockdown. A-B. Immunoblot of MKL-1 cells stably expressing GFP, TIP60 α (A.), or TIP60 β (B.) infected with lentivirus containing either a control shRNA or a TIP60-specific shRNA. WT = wild type TIP60; mut = catalytic dead TIP60 (Q377E and G380E). C-D. Cell viability of MKL-1 cell lines from A. and B. after shRNA expression via lentivirus infection as measured by CellTiter-Glo® assay and normalized to shCtrl at each time point. Top panel: cells expressing either a shTIP60-1 resistant TIP60 or GFP were infected with shTIP60-1 lentivirus. Bottom panel: cells expressing either a shTIP60-6 resistant TIP60 or GFP were infected with shTIP60-6 lentivirus. Error bars represent SD. N=2.

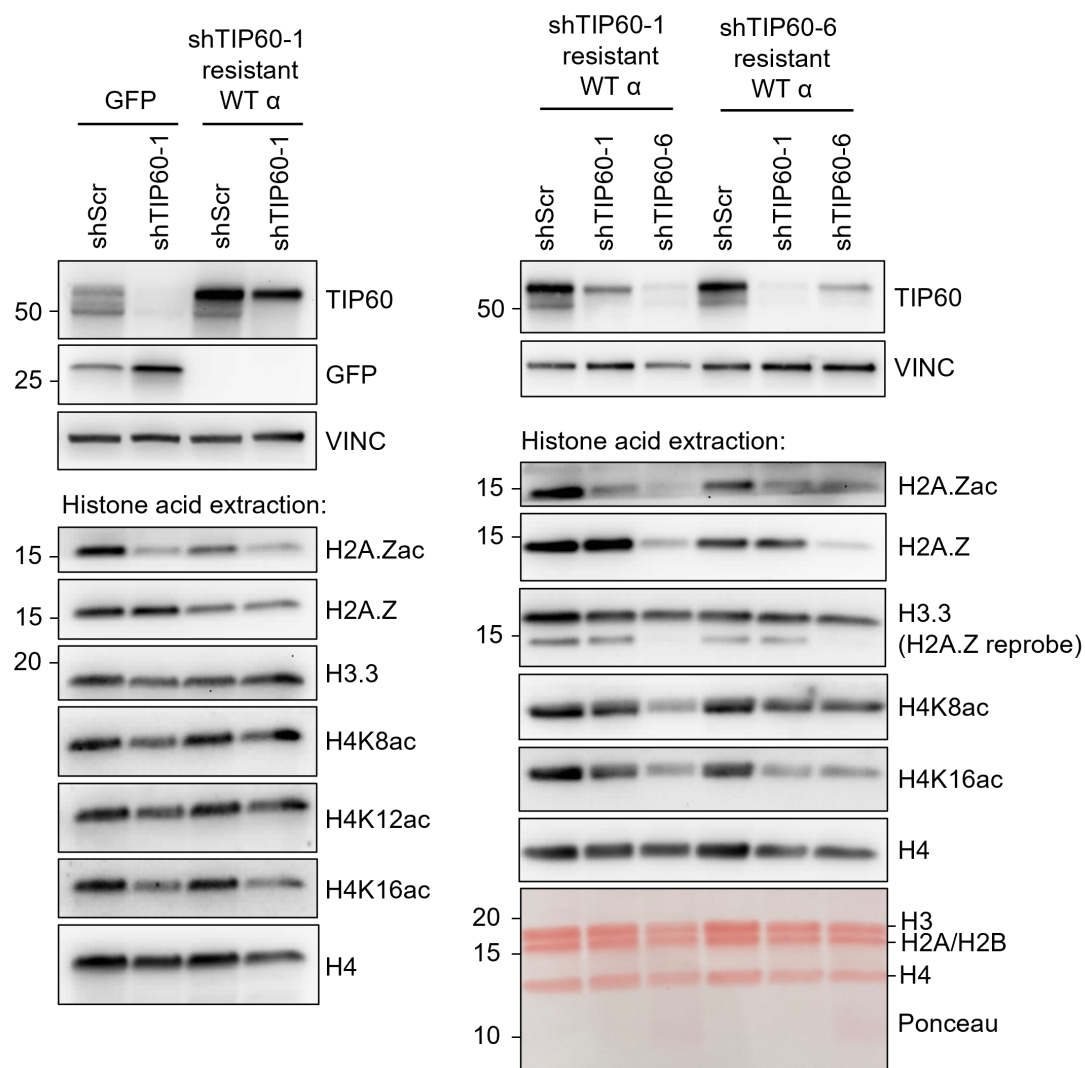


Figure 2.4: Overexpression of TIP60 α is not sufficient to rescue histone acetylation loss with TIP60 knockdown. Immunoblots of total protein lysate (top) and acid extracted histones (bottom) for MKL-1 cells expressing GFP, shTIP60-1 resistant TIP60 α , or shTIP60-6 resistant TIP60 α . Cells were collected for immunoblot 5 days after infection with lentivirus containing indicated shRNA. Left: N=1; Right: N=3.

and TIP60 β (Figure 2.5A). We found that overexpression of both TIP60 major isoforms was not able to rescue H2A.Zac or H4K16ac loss with TIP60 knockdown. Instead, we found that overexpression of both major isoforms together led to decreased levels of H4K16ac and total H2A.Z. We also asked whether overexpression of both isoforms provided resistance to TIP60 knockdown (Figure 2.5B). We found that there was a slight increase in viability of cells expressing shRNA-resistant TIP60 isoforms treated with shTIP60-1 but this did not reach statistical significance. There was no survival benefit in TIP60-expressing cells treated with shTIP60-6.

2.4.2 H2A.Z AND H2A.ZAC MARKS CORRELATE WITH SLAP COMPLEX BINDING AT GENE PROMOTERS.

Out of all the histone acetylation marks tested, the effect of TIP60 knockdown on H2A.Z acetylation was the most striking. H2A.Z deposition has been associated with P400 activity^{126,127,128}, so we hypothesized that the TIP60/P400 complex remodeled chromatin at L-MYC target genes through deposition and acetylation of H2A.Z. To address this hypothesis, we first performed ChIP-qPCR of H2A.Z and H2A.Zac using primers specific to two SLAP target genes, BRWD1 and MDM2, and two non-target genes, one actively expressed (GAPDH) and one repressed (KRT9) (Figure 2.6A). We also included an antibody specific for H3K4me3, a mark of active gene promoters that is also enriched at MYC target genes^{132,143} as a control. We found that H3K4me3 was enriched at all three highly-expressed genes BRWD1, MDM2, and GAPDH while H2A.Z and H2A.Zac were only enriched at BRWD1 and MDM2.

ChIP-qPCR is limited to a finite number of target genes, so to determine whether H2A.Z and H2A.Zac correlate with SLAP complex binding across the genome we performed ChIP-seq using H2A.Z and H2A.Zac antibodies. We compared the binding

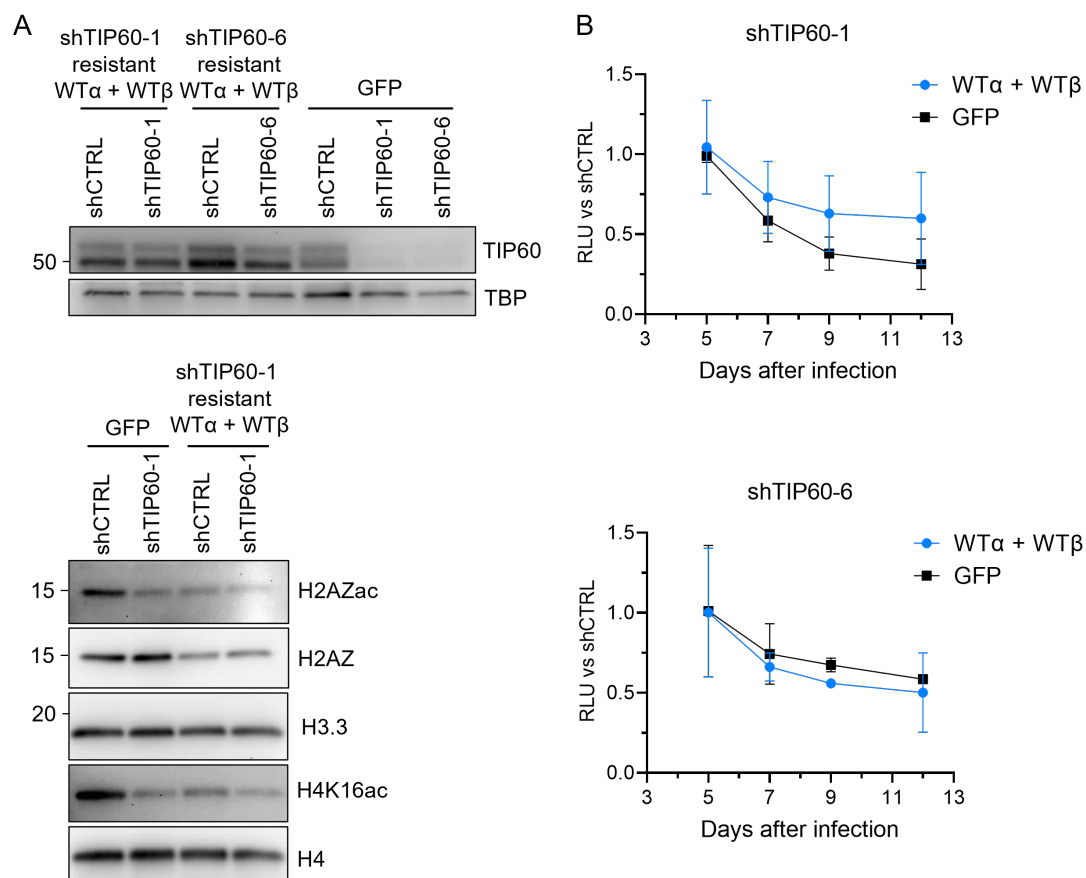


Figure 2.5: Overexpression of TIP60 α and TIP60 β is not sufficient to rescue cell viability or histone acetylation loss with TIP60 knockdown. A. immunoblots of total protein lysate (top) and acid extracted histones (bottom) for MKL-1 cells expressing GFP, shTIP60-1 resistant TIP60 α + TIP60 β , or shTIP60-6 resistant TIP60 α + TIP60 β and infected with indicated shRNA. Cells were collected on day 12. **B.** Cell viability measured by CellTiter-Glo® assay of MKL-1 cells expressing both TIP60 α + TIP60 β and infected with lentivirus expressing indicated shRNA. Error bars = SD. N=2.

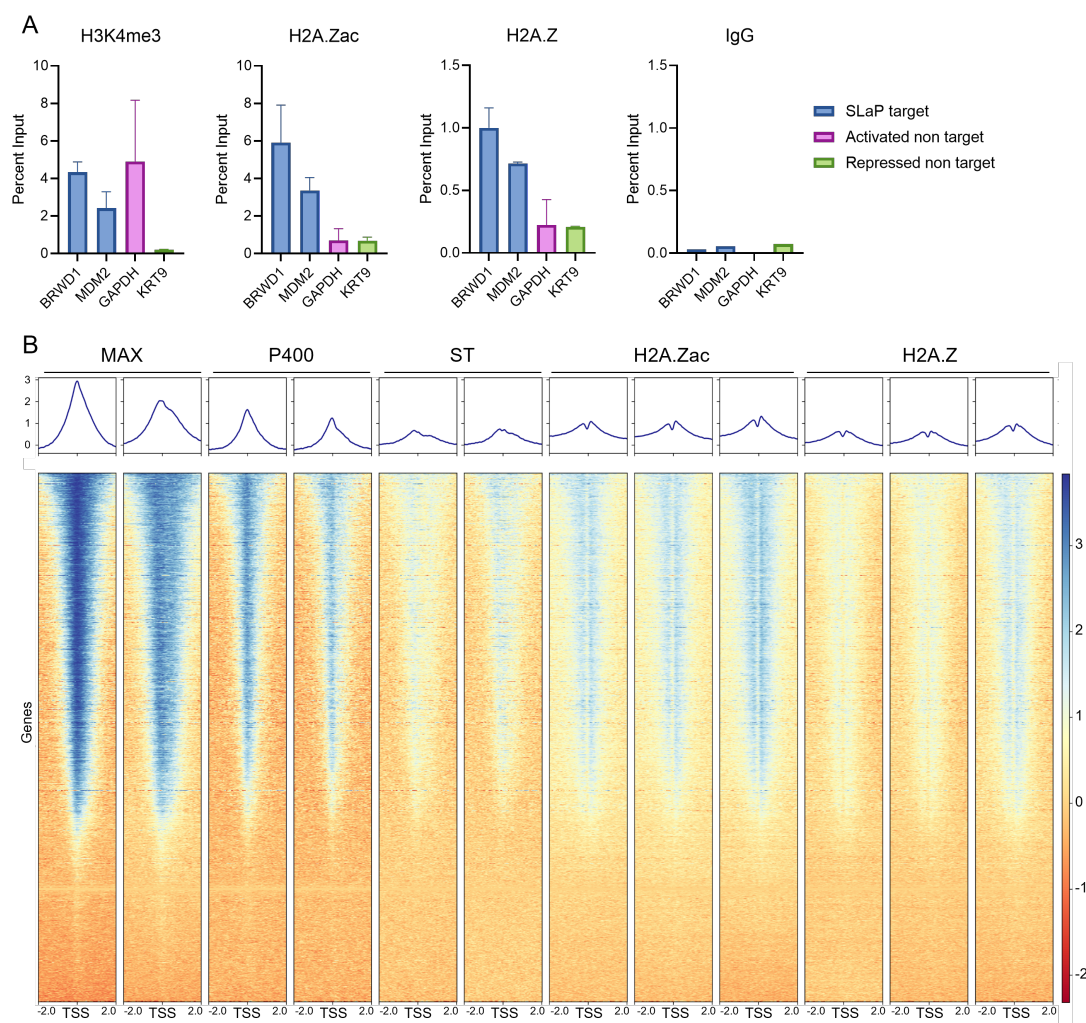


Figure 2.6: Histone marks associated with TIP60/P400 activity correlate with SLaP complex chromatin binding. **A.** ChIP-qPCR of histone marks H3K4me3, acetylated H2A.Z (H2A.Zac), and total H2A.Z in MKL-1 cells using primers specific to previously identified SLaP target genes (BRWD1 and MDM2), a highly expressed non-SLaP target gene (GAPDH), and a lowly-expressed/repressed non-SLaP target gene (KRT9). N=2. **B.** ChIP-seq of H2A.Z and H2A.Zac was performed and compared with ChIP-seq results for SLaP complex components MAX, P400, and MCV ST from⁴⁸.

profiles to previously reported SLaP binding data in MKL-1 cells for MAX, P400, and ST⁴⁸ (Figure 2.6B). We focused on specifically gene TSSs as the SLaP complex was previously reported to predominantly bind at TSSs. We found that both H2A.Z and H2A.Zac almost completely overlapped with MAX, P400, and ST binding across the genome. These findings are in agreement with our model that the catalytic activities of TIP60 and P400 are active in the SLaP complex and may contribute to an active chromatin state at SLaP target gene promoters.

2.5 DISCUSSION

Here, we investigate the role of TIP60 in regulating the chromatin context surrounding the transcriptional start sites of SLaP target genes in MCC. We find that all four previously reported TIP60 isoforms are not only expressed in MCC cells but that they can all co-precipitate with components of the SLaP complex, including T antigens. Using shRNA targeting all TIP60 isoforms we found that TIP60 knockdown correlated with cell viability loss in both MCCP and MCCN cells. Even though we would not expect MCCN cells to express T antigens and therefore would not contain a SLaP complex, it would not be entirely surprising to find that TIP60 is also essential in these cells. TIP60 is considered an essential gene in 1059 out of 1078 CRISPR screens in cancer cell lines according to data from the Dependency Map portal by the Broad Institute (<https://depmap.org/portal/gene/KAT5?tab=overview>). Although this finding does highlight the importance of TIP60 activities in cancer cell biology, it introduces additional challenges to studying TIP60 function as generating stable TIP60 knockout lines isn't feasible.

We next generated TIP60 α and TIP60 β rescue lines in an effort to study the in-

dependent contributions of TIP60 isoforms. We expected that overexpression of the canonical isoform TIP60 α would provide some rescue of the cell viability lost with TIP60 knockdown. To our surprise, neither TIP60 α nor TIP60 β expression alone provided any cell viability benefit. We therefore tried expressing both major TIP60 isoforms together before inducing TIP60 knockdown, but this strategy provided only a slight survival benefit that didn't reach significance for either shRNA. It's possible that all four TIP60 isoforms are essential in MCC cells, but another explanation for these findings is that both TIP60 shRNA have off target effects that are impacting cell viability independently of TIP60 expression. In fact, the *KAT5* gene overlaps with the *RNASEH2C* gene so that the 3' end of both genes overlap, *KAT5* on the positive strand and *RNASEH2C* on the negative strand. Although the guide strand for shTIP60-1 is not complementary to *RNASEH2C*, the passenger strand is a perfect match and could be affecting *RNASEH2C* expression. Strategies to reduce passenger strand effects include designing shRNA with shorter target sequences (18 nts) and shorter loops (<5 nts)¹⁴⁴. This theory would need to be tested experimentally but at the very least this study highlights the necessity of precise guide design to reduce the likelihood of passenger strand off target effects and the importance of performing rescue experiments to ensure that any shRNA effects are specific to the target gene.

Although TIP60 overexpression did not rescue cell viability loss, we wanted to determine whether we could detect changes in histone modifications with TIP60 knockdown that could be rescued with overexpression. Given that TIP60 has previously been reported to acetylate histones H4 and H2A.Z, we induced TIP60 knockdown and collected samples using histone acid extraction followed by immunoblot. We found that loss of TIP60 noticeably affected levels of H2A.Z and H4K16 acetylation but had little to no effect on H4K8 or H4K12 acetylation. This finding is in alignment

with previous work but surprisingly rescue with either TIP60 α alone or with both major TIP60 isoforms had little to no effect. One of the shRNA, shTIP60-6, actually reduced total levels of H2A.Z independently of TIP60 expression. Interestingly, overexpression of TIP60 α or TIP60 α and β reduced both total H2A.Z and H4K16ac levels suggesting that high levels of TIP60 can actually negatively regulate H2A.Z expression and H4K16 acetylation. H2A.Z was not previously identified as a SLaP target gene, however ST, P400, and MAX peaks can be identified near the *H2AZI* transcriptional start site, along with H2A.Z and H2A.Zac peaks (Figure 2.7), so it is possible that the SLaP complex, or at least the TIP60/P400 complex, regulates total H2A.Z expression.

But why would higher levels of TIP60 reduce H2A.Z expression? One possible explanation is that the SLaP complex activates expression of a protein that negatively regulates H2A.Z expression, such as a transcriptional repressor. There is already precedence for the SLaP complex activating the LSD1-CoREST repressor complex⁶³, although there is no evidence that this repressor complex targets *H2AZI* (Figure 2.7). Another possible explanation is that TIP60 itself can repress gene expression. Acharya et al. found that TIP60 can repress gene expression in a lysine acetyltransferase-independent manner in mouse embryonic stem cells¹⁴⁵. They propose that TIP60/P400 binding can prevent the recruitment of additional transcriptional activators. We also saw that H4K16ac levels decreased with overexpression of the major TIP60 isoforms, indicating that high levels of TIP60 can have a dominant negative effect on acetylation. Further work is necessary to understand how high levels of TIP60 directly or indirectly repress H4K16 acetylation and H2A.Z expression.

H2A.Z deposition and acetylation by P400 and TIP60 respectively has been previously reported by multiple groups as a mechanism to activate gene expression. Be-

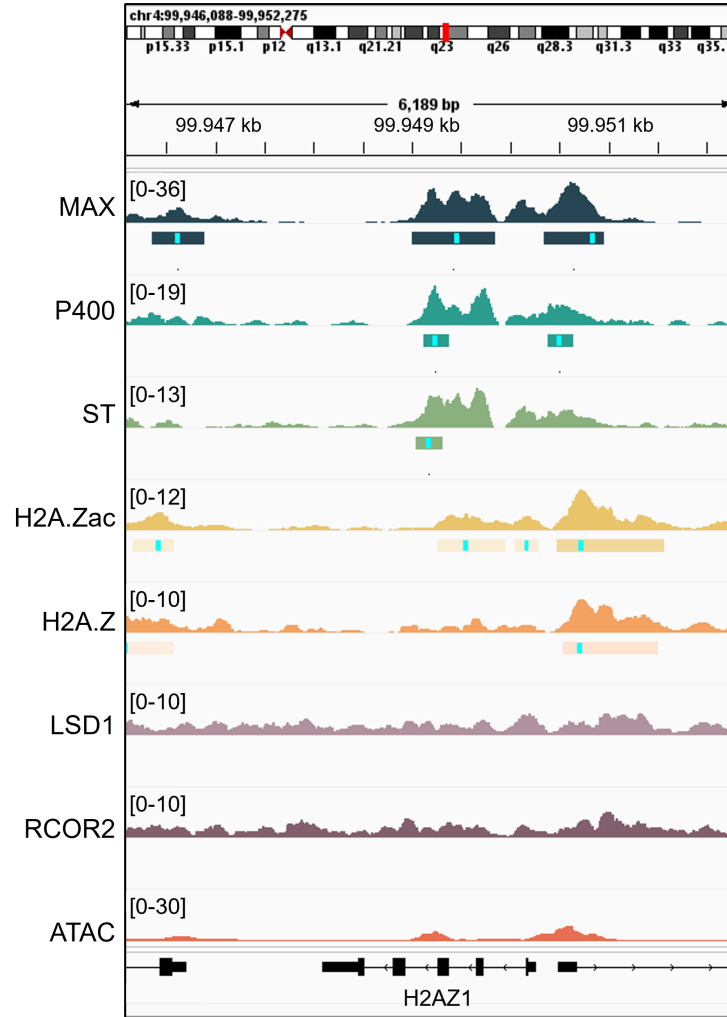


Figure 2.7: The SLaP complex, but not the LSD1-CoREST complex, is enriched at the *H2AZ1* promoter. Fold change of read pileups from pooled replicates of ChIP-seq or ATAC-seq data compared to background at the *H2AZ1* transcriptional start site. Statistically significant ChIP-seq peaks are marked with bars underneath. ChIP-seq data for MAX, P400, and ST from Cheng et al.⁴⁸, ChIP-seq data for LSD1 and RCOR2 from Park et al.⁶³, and ATAC-seq data from Park et al.⁶³ was reanalyzed using the ENCODE transcription factor ChIP-seq or ATAC-seq analysis pipeline respectively. N=3 for histone ChIP-seq.⁴⁸

cause our data with TIP60 knockdown and rescue was somewhat confounded by off target effects and unexpected results with greater than physiological levels of TIP60, we wanted to determine whether this mechanism of gene activation could be relevant in MCC. We performed ChIP-seq of H2A.Z and H2A.Zac and compared the binding profiles with that of the SLaP complex. We found that the binding profiles overlapped almost completely. This finding is in support of the model that TIP60 and P400 activities promote incorporation of H2A.Z and acetylated H2A.Z into chromatin around transcriptional start sites to promote active gene expression. Additional work is necessary to determine whether this correlation has mechanistic significance for gene trans-activation by the SLaP complex.

AUTHOR CONTRIBUTIONS FOR CHAPTER 3

The PRMT5 inhibitor dose curve experiments in MCCP and MCCN cell lines were designed by Jingwei Cheng and performed by Camille H. Cushman. The cell cycle distribution flow cytometry experiments were performed by Camille H. Cushman with guidance from Hembly G. Rivas. The dose curves and synergy experiments in Figure 3.7 were performed by Hellena Vallee. All other experiments were conceptualized and performed by Camille H. Cushman with input from James A. DeCaprio.

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3

Defining the role of p53 activity in sensitivity to PRMT5 inhibitor in MCC.

3.1 ABSTRACT

Merkel cell carcinoma is a highly malignant skin cancer with an unmet clinical need warranting further investigation into new treatment strategies. In this chapter, we identified the arginine methyltransferase PRMT5 as a potential therapeutic target in MCC cells and demonstrated that both established and patient-derived MCC cell lines have

reduced viability when treated with PRMT5 inhibitor in a dose- and time-dependent manner. We evaluated potential markers of PRMT5 inhibitor sensitivity and found that neither PRMT5 expression nor levels of symmetric dimethylarginine, the mark deposited by PRMT5, correlated with sensitivity to PRMT5 loss. We showed that PRMT5 inhibition activated a p53 response and induced cell cycle arrest and apoptosis. Using genetic methods to inhibit p53 activity, we showed that p53 was not necessary for PRMT5 inhibitor-mediated cell cycle arrest or cell death indicating that alternative factors are important for PRMT5 inhibitor sensitivity.

3.2 INTRODUCTION

Merkel cell carcinoma is an aggressive cancer with a low overall survival rate and a high likelihood for metastasis. Despite advances in treatments with the approval of multiple immune checkpoint blockade therapies for late stage and refractory MCC, many immunocompromised MCC patients are unable to receive these treatments due to contraindications (reviewed in²⁴). Among those that can be treated, about 50% will have either intrinsic or acquired resistance highlighting a need for continued investigation into alternative treatment strategies.

Previous efforts to characterize molecular mechanisms of MCC development led to the identification of LSD1 and MDM2 inhibitors as potential treatment strategies^{67,63}, and MDM2 inhibitors are currently being investigated in the clinic (NCT03787602). These proteins were identified as downstream targets of the SLAP complex, a transcriptional regulatory complex thought to transactivate L-MYC target gene expression in MCCP tumors⁴⁸. By re-examining the list of previously identified SLAP target genes⁴⁸, we noticed that at least 4 members of the protein arginine methyltransferase

(PRMT) family of enzymes were also transcriptional targets, including PRMT1, 3, 5, and 7. By integrating our H2A.Z and H2A.Zac data from Chapter 2 with SLaP complex binding data from Cheng et al.⁴⁸, we see evidence for both SLaP complex binding and histone acetylation at the transcriptional start sites of all four PRMTs (Figure 3.1).

PRMT1 and 5 are among the PRMTs the most highly expressed in cancer with higher expression correlating with a worse prognosis (reviewed in⁷⁹), so it is possible that SLaP-mediated activation of these genes has important functional roles in MCCP oncogenesis. PRMT5 in particular catalyzes symmetric dimethyl arginine (SMDA) residues and has been the subject of numerous research and drug discovery efforts. A number of PRMT5-specific inhibitors have entered into clinical trials (NCT02783300, NCT03614728, NCT04676516, NCT03573310, and NCT03854227). Notably, an analysis of 240 cancer cell lines treated with the PRMT5 inhibitor GSK-3203591 found that PRMT5 inhibitor sensitivity correlated with *TP53* status, with *TP53* wild type (WT) cancer significantly more likely to be sensitive⁷⁵. Many groups have found that PRMT5 loss triggers a p53 response in diverse cell types and model systems including: mantle cell lymphoma (MCL), breast cancer, B cell acute lymphoblastic leukemia, B cell lymphoma⁷⁵; melanoma⁷⁷; hematopoietic cells⁷⁶; and in a mouse conditional PRMT5 knockout (KO) model⁷⁴. These findings are of particular interest to us as many MCCP tumors are *TP53* WT, and previous work has shown that reactivating p53 activity is a promising therapeutic strategy in MCCP⁶⁷.

Multiple groups have found that PRMT5 can regulate p53 activity through MDM4 splicing as PRMT5 has been shown to directly methylate components of the spliceosome to regulate alternative splicing (reviewed in^{78,80}). MDM4 (or MDMX) has p53 binding activity and can either inhibit p53 gene transactivation or can complex with

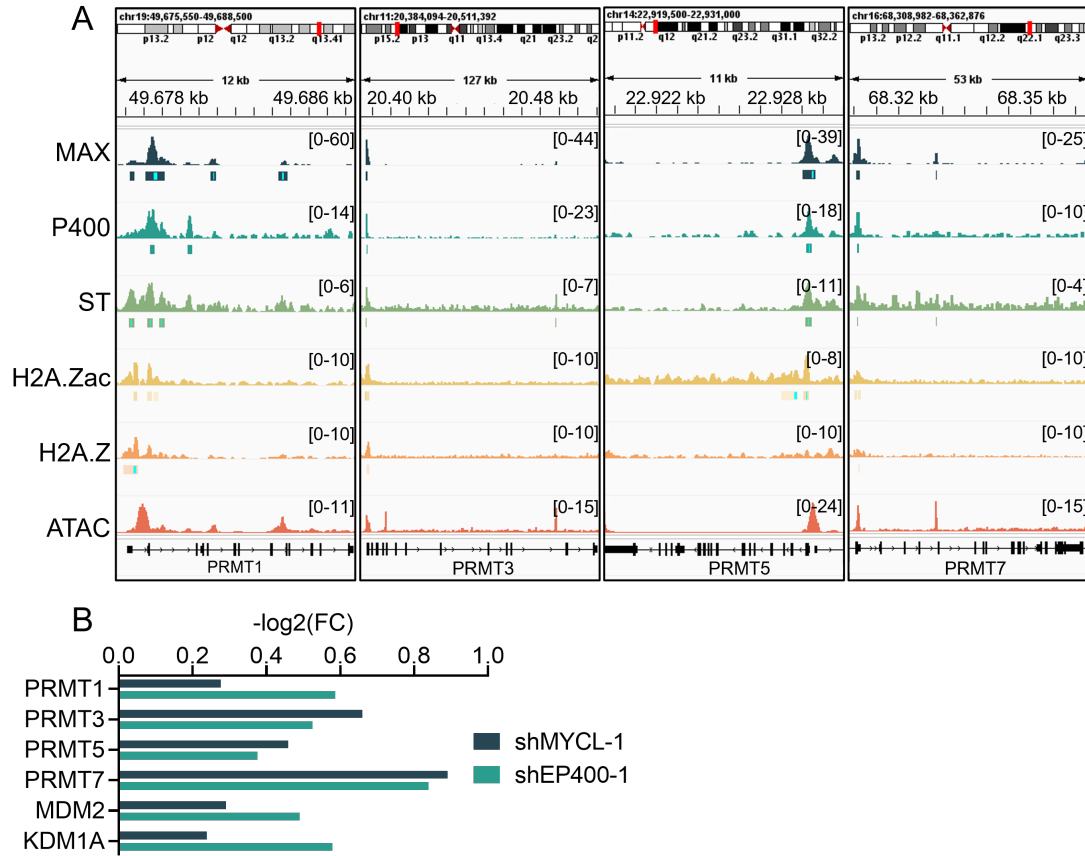


Figure 3.1: The SLaP complex activates expression of PRMTs. **A.** Fold change of read pileups from pooled replicates of ChIP-seq or ATAC-seq data from MKL-1 cells compared to background at the gene transcriptional start sites of PRMT1, 3, 5, and 7. Statistically significant ChIP-seq peaks are marked with bars underneath. ChIP-seq data for MAX, P400, and ST from Cheng et al.⁴⁸ and ATAC-seq data from Park et al.⁶³ were reanalyzed using the ENCODE transcription factor ChIP-seq or ATAC-seq analysis pipeline respectively. **B.** Negative log₂ fold change of transcripts for the indicated SLaP target genes including PRMT5 as measured by RNA-seq compared to an shRNA control in MKL-1 cells from Cheng et al.⁴⁸.

MDM2 to promote p53 degradation (reviewed in⁶⁹). Many groups have shown that PRMT5 loss through either gene KO or PRMT5 inhibitor treatment leads to exon skipping of MDM4 exon 6 and reduced MDM4 protein expression^{74,75,76,77}. Bezzi et al. in particular propose that MDM4 splicing is a mechanism by which p53 can sense splicing defects⁷⁴ and in fact knockdown of many different individual splicing factors leads to MDM4 loss and p53 activation¹⁴⁶.

The significance of p53 activation with PRMT5 loss has been debated, with some studies arguing that p53 plays an important role in sensitivity to PRMT5 inhibition whereas others assert that p53 activity plays only a minor role. Gerhart et al. found that overexpression of MDM4 in an MCL line or p53 KO in a colorectal adenocarcinoma line reduced sensitivity to GSK-3203591, a substrate-competitive PRMT5 inhibitor⁷⁵. Erazo et al. demonstrated similar results by identifying *TP53* loss as a marker of resistance in a CRISPR screen performed in two lymphoma lines¹⁴⁷. They went on to show that overexpression of p53 in a *TP53* mutant and GSK-3203591-resistant MCL line rescued sensitivity to PRMT5 inhibition.

By contrast, Scoumanne et al. report that p53 loss is not sufficient to rescue cell viability defects with PRMT5 loss in a breast cancer cell line¹⁴⁸. Braun et al. found no significant correlation between PRMT5 inhibitor sensitivity and p53 status across 25 different cancer cell lines⁹⁹, and Gerhart et al. acknowledge that additional mechanisms must contribute to PRMT5 inhibitor sensitivity as there were a number of p53 mutant, PRMT5 inhibitor-sensitive lines⁷⁵. Hamard et al. found that in a PRMT5 KO mouse model, p53 KO only partially rescued the PRMT5 loss phenotype⁷⁶.

Altogether, the results of these studies suggest that PRMT5 loss triggers a p53 response, but how important that response is to mediating PRMT5 inhibitor sensitivity likely depends on the cancer type. Here, we investigate the effect of PRMT5 in-

hibition on both p53 WT and p53 mutant MCC lines. We find that p53 status correlates with PRMT5 inhibitor sensitivity in MCCC cell lines, but that MCCN cell lines are sensitive to the inhibitor independently of p53 status. We find that the effect of PRMT5 inhibitor on MCC viability is dependent on both time of treatment and dose of inhibitor. We show that MDM4 expression is lost and p53 is activated at early time points with PRMT5 inhibition, correlating with or preceding cell cycle arrest and apoptosis in p53 WT MCCC lines. We also show that PRMT5 inhibition in combination with MDM2 inhibition is more effective than single-drug treatment. We find however that knockout of p53 or expression of a dominant negative p53 mutant provides no resistance to PRMT5 inhibition and has little to no effect on the cell cycle arrest and apoptotic phenotypes we observed. We conclude that although PRMT5 inhibitor treatment activates a p53 response in MCC, this response is not required for sensitivity.

3.3 METHODS

3.3.1 CELL CULTURE

ESTABLISHED MCC LINES

MCC cell lines WaGa, PeTa, MKL-1, BroLi, MS-1, MKL-2, UISO, MCC26, and MCC13 were gifts from Masahiro Shuda (University of Pittsburgh), Jürgen Becker (Translational Skin Cancer Research, German Cancer Consortium (DKTK), Essen, Germany), and Roland Houben (University of Wuerzburg, Germany). Cells were cultured in RPMI (Life Technologies, 11875119) with 10% FBS and 1% penicillin-streptomycin (Life Technologies, 15140163). Cell lines were confirmed to be free of mycoplasma contamination using the e-Myco Mycoplasma PCR Detection Kit (Bull-

dog Bio, 25233).

P53 knockout (p53KO 1-1, 1-2, and 1-3) and control (non-targeting control and AAVS1) MKL-1 cell lines were generated previously using a CRISPR/Cas9 gene knockout strategy⁷³. WaGa cell lines expressing doxycycline-inducible p53DD or GFP in the pLIX-402 backbone (Addgene, 41394) were generated previously using lentiviral transduction¹.

PATIENT-DERIVED MCC LINES

MCC350 and MCC336 were generated in house from patient tumors as described^{149,150,73}. Cells were cultured in NeuroCult™ NS-A media (StemCell Technologies, 05750) with 10% proliferation supplement (StemCell Technologies, 05753) supplemented with 20 ng/mL human recombinant bFGF (StemCell Technologies, 78003.1), 20 ng/mL human recombinant EGF (LifeTechnologies, PHG0311), and 0.02% heparin (StemCell Technologies, 07980).

3.3.2 CELL VIABILITY ASSAYS

Cells were plated into 100 uL drug-containing growth media in opaque 96-well plates. Suspension cells (WaGa, PeTa, MKL-1, BroLi, MS-1, MKL-2, MCC350, and MCC336) and UISO cells were plated at a density of 1,000 cells/well while MCC13 and MCC26 cells were plated at a density of 100 cells/well into PRMT5i-containing media. An additional 1x volume of drug-containing media was added to each well on day 4. For WaGa cell lines expressing doxycycline (dox)-inducible p53DD or GFP, cells were treated with 1 ug/mL dox 1 day before being plated into 96-well plates containing both 1 ug/mL dox and PRMT5i, which was added again on day 4. Cell viability was assessed on day 8 using the CellTiter-Glo® Luminescent Cell Viability Assay

(Promega, G7571) according to the manufacturer's instructions with the following modifications: 25 uL of CellTiter-Glo® reagent was added per 100 uL sample (50 uL total) and was incubated at RT on a shaker for 12 min prior to quantification.

3.3.3 IMMUNOBLOT

Cell pellets were lysed in EBC buffer and protein content was quantified by Bradford assay. Lysates were separated on a 4-20% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked in 5% milk, and incubated with primary antibody at 4°C overnight. A list of the primary antibodies used can be found in Table 3.1. Blots were imaged with either SuperSignal West Pico (ThermoFisher, PI34080) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500).

Table 3.1: Antibodies used for immunoblot

Target	Catalog Number
GFP	CST 2956S
MDM4	Abcam ab243859
p21	CST 2946S
p53	CST 2527S
p53 C-terminus (for p53DD)	Santa Cruz SC-393031
PRMT5	Santa Cruz SC-376937
SDMA	CST 13222S
TBP	CST 8515S

3.3.4 ANNEXIN V/PI STAINING

Annexin V and PI staining was performed using the BD Annexin V: FITC Apoptosis Detection Kit I (Fisher Scientific, BDB556547) according to the manufacturer's instructions. Samples were analyzed by flow cytometry (BD LSRFortessa) at the Dana-Farber flow cytometry hematologic neoplasia and Jimmy fund core.

3.3.5 CELL CYCLE DISTRIBUTION

Cell cycle distribution was determined as previously described¹⁵¹ without the primary or secondary antibody incubation steps. Briefly, cells were treated with 10 μ M EdU (Click Chemistry Tools, 1149-100) for 1 hour prior to collection. Cells were fixed in 4% formaldehyde in PBS for 15 minutes at room temperature before being permeabilized in 70% ethanol at -20°C overnight. Incorporated EdU was labeled using a CLICK reaction (2 mM CuSO₄, 100 M THPTA, 100 mM sodium ascorbate, and 2 M Alexa 647 Azide in PBS) by rotating for 30 minutes at room temperature protected from light. DNA was stained with a 1 μ g/mL DAPI, 100 ng/mL RNase A staining solution for 1 hour at room temperature protected from light prior to analysis by flow cytometry (BD LSRFortessa) at the Dana-Farber flow cytometry hematologic neoplasia and Jimmy fund core.

3.3.6 SYNERGY STUDIES

Cells were plated into 96-well plates at a concentration of 1×10^5 cells/mL in 100 μ L of RPMI growth media. PRMT5i (JNJ-64619178) and nutlin-3A doses were added using a HP D300e digital dispenser at the Harvard medical school ICCB-Longwood screening facility. For 8 day treatments, an additional 1X volume of RPMI with inhibitor was added after 4 days. Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega G7571) according to the manufacturer's instructions with the following modifications: 25 μ L of CellTiter-Glo® reagent was added per 100 μ L sample and was incubated at RT on a shaker for 12 min prior to quantification.

3.4 RESULTS

3.4.1 MCC SENSITIVITY TO PRMT5 INHIBITORS.

Sensitivity to PRMT5 inhibitors has previously been shown to correlate with p53 status, with p53 WT cancers more likely to be sensitive to loss of PRMT5. Given that many MCCC tumors are p53 WT, we hypothesized that PRMT5 inhibition would be toxic to MCC lines in a p53-dependent manner. To address this hypothesis, we treated a panel of MCCC cell lines with the PRMT5 inhibitor JNJ-64619178, a potent PRMT5 inhibitor currently in phase 1 clinical trials (NCT03573310) (Figure 3.2). We included four p53 WT cell lines and two p53 mutant (mut) cell lines and found that all p53 WT lines were sensitive to this PRMT5 inhibitor while the two p53 mut lines were more resistant. We then treated a panel of MCCN lines as well and found that all MCCN lines were highly sensitive to PRMT5i regardless of p53 status. Similarly,

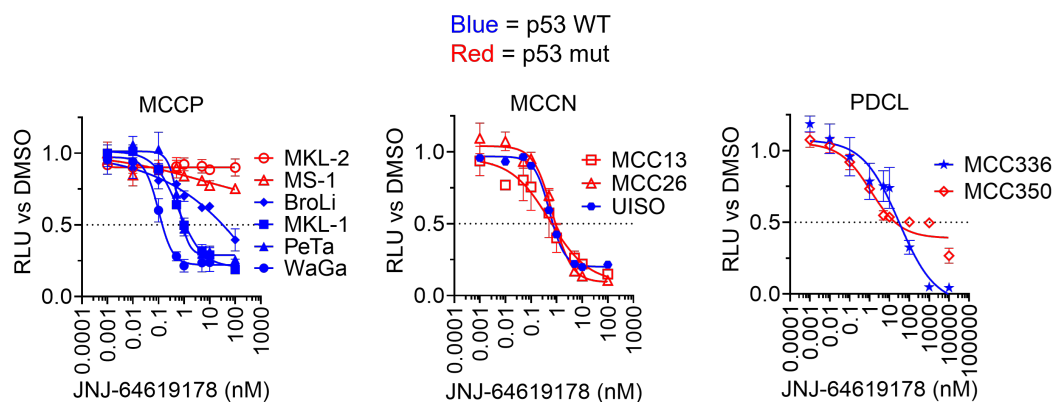


Figure 3.2: Sensitivity to PRMT5 inhibitors correlates with TP53 status in MCCC, but not MCCN. MCCC (left), MCCN (center), and patient-derived cell lines (PDCL; right) were treated with increasing doses of the PRMT5 inhibitor JNJ-64619178 (PRMT5i) and cell viability was measured by CellTiter-Glo® after 8 days. Cell lines are colored by p53 status: p53 wild-type in blue and p53 mutant in red. MCC350 = MCCN; MCC336 = MCCC. N=3.

Table 3.2: MCC sensitivity to PRMT5i JNJ-64619178 with 8 day treatment

Cell Line	GI50 (nM)	Viral status	p53 status
WaGa	0.141	+	+
MCC13	0.647	-	S241F; P278S
UISO	0.718	-	+
PeTa	0.896	+	+
MCC26	0.901	-	T125T (splice)
MKL-1	1.102	+	+
MCC350	19.479	-	V218G
MCC336	30.675	+	+
BroLi	32.908	+	+
MS-1	>100	+	251-253 del
MKL-2	>100	+	FS del

we treated two patient-derived cell lines (PDCL) with JNJ-64619178, one MCCN (MCC350) and one MCCP (MCC336) and found that both were similarly sensitive to the inhibitor regardless of p53 status. Comparing the 50% growth inhibitory concentrations (GI50) across cell lines (Table 3.2), we see that the most sensitive MCC lines have sub-nanomolar GI50 concentrations with WaGa cells being the most sensitive and MS-1 and MKL-2 cells being the least sensitive. These findings suggest that p53 may play a role in sensitivity to PRMT5 loss in MCCP cells, but in MCCN cells PRMT5 activity is essential regardless of p53 status.

We asked whether this finding was reproducible by treating a subset of the MCCP lines using a PRMT5 inhibitor that has entered into phase 2 clinical trials, GSK-3326595 (NCT04676516; Figure 3.3). GSK-3326595 is a PRMT5 inhibitor with a different mechanism of action from JNJ-64619178. GSK-3326595 competes for binding with PRMT5 substrates⁷⁵ while JNJ-64619178 binds to the SAM binding pocket¹⁵². We found that the sensitivity of WaGa, MKL-1, and MS-1 cells corroborated the results from Figure 3.2 with WaGa cells being the most sensitive and MS-1 cells resistant.

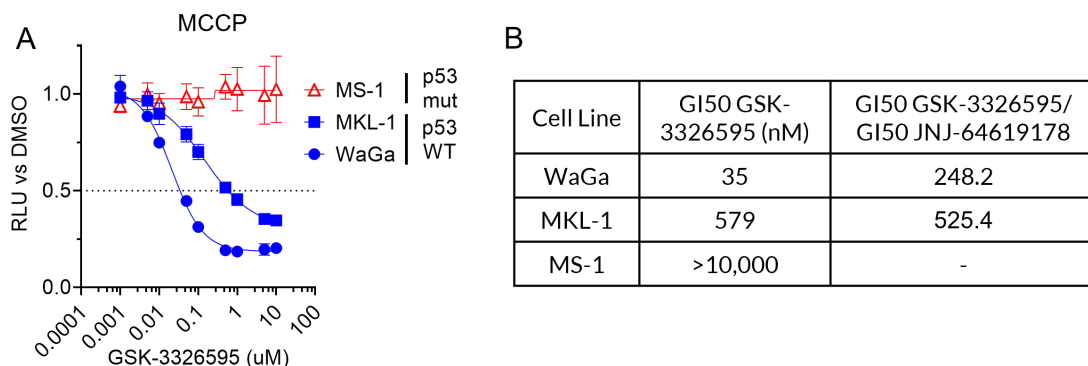


Figure 3.3: JNJ-64619178 is a more potent PRMT5 inhibitor than GSK-3326595. A. MCCP cells were treated with increasing doses of the PRMT5 inhibitor GSK-3326595 and cell viability was measured by CellTiter-Glo®. Fresh PRMT5i-containing media was added on day 4 and viability was measured on day 8. N=3. **B.** Table of GI50 values for MCCP cell lines treated with GSK-3326595 compared with GI50 values for JNJ-64619178 from Figure 3.2

Importantly, the GI50 concentrations with GSK-3326595 were about 250-525 times greater than for JNJ-64619178. Given that the JNJ-64619178 inhibitor was much more potent than the GSK-3326595 drug, we decided to focus our efforts using the JNJ-64619178 inhibitor which we refer to as PRMT5i.

Luminescent-based cell viability assays are a useful tool for measuring drug effects, however they are limited in that they cannot distinguish cell growth arrest from cell death. To get a better sense as to how PRMT5i affected MCC cell viability over dose and time, we performed a cell counting assay using WaGa cells treated with varying doses of PRMT5i over a 12 day time course (Figure 3.4). We found that there was an effect on cell count with as low as 0.5 nM PRMT5i treatment that reached statistical significance by 8 days ($p < 0.0001$), however at this dose PRMT5i had an affect on cell growth rate and not cell death as the total cell count was still increasing over time. At doses of 5 nM or above, cell counts increased between 0 and 4 days at a sig-

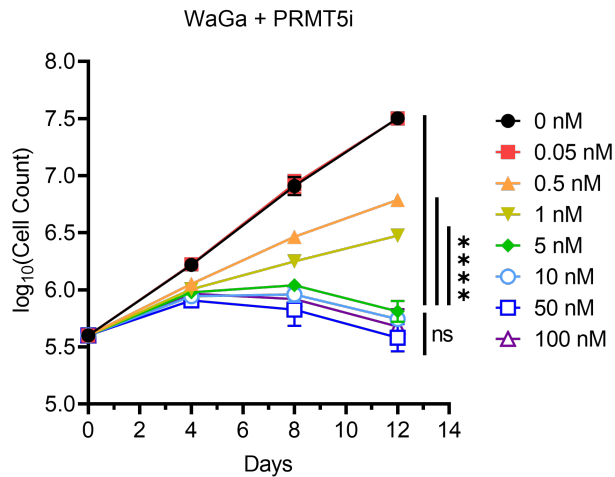


Figure 3.4: PRMT5i has both a dose and time dependent effect on cell growth. Cell counting assay of WaGa cells treated with varying doses of PRMT5i over 12 days. Cells were counted and drug was refreshed every 4 days. Statistics describe comparisons of each concentration vs 5 nM at 12 days by two-way ANOVA using Tukey's multiple comparisons test. ns = not significant; **** = $p < 0.0001$. N=3.

nificantly slower rate than DMSO, stayed relatively consistent between 4 and 8 days, before decreasing between 8 and 12 days. There was no statistically significant differences identified between 5, 10, 50, and 100 nM treatments at any time point tested ($p > 0.05$), however the difference between DMSO and PRMT5i treatment at 4 days only reached statistical significance with doses greater than 5 nM (DMSO vs 5 nM, $p = 0.0955$; 10 nM $p = 0.0347$; 50 nM $p = 0.0092$). We decided to use 10 nM for many of our assays going forward. We conclude that the effect of PRMT5i on cell growth is both time- and dose-dependent.

After establishing that MCC cells are sensitive to PRMT5 inhibition, we wanted to assess likely determinants of sensitivity in an effort to identify useful biomarkers for treatment consideration. We first examined the levels of PRMT5 and SDMA across MCC cell lines with and without PRMT5i treatment (Figure 3.5). We found

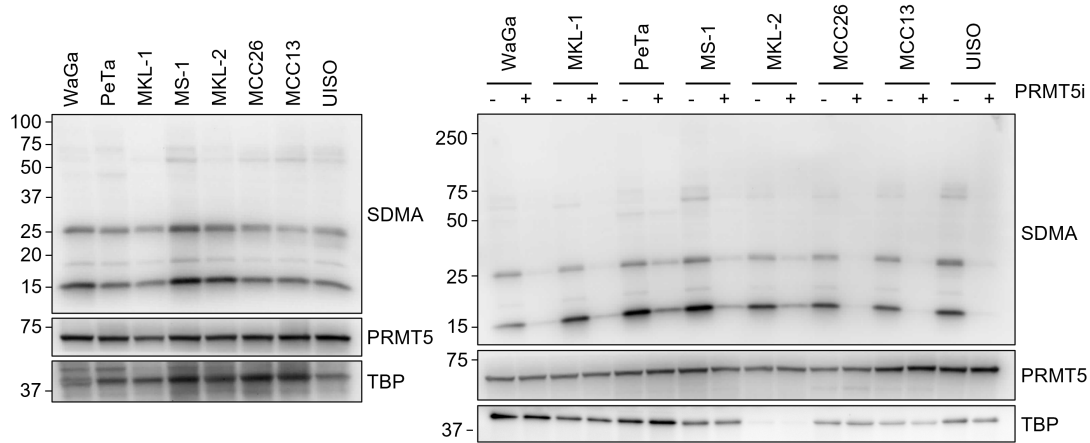


Figure 3.5: PRMT5i sensitivity of MCC cells is independent of PRMT5 or SDMA levels. Immunoblots of MCC cells either untreated (left) or treated (right) with 10 nM PRMT5i for 4 days. Representative of 3 images.

that PRMT5 levels were very consistent between cell lines while there was greater variation in SDMA levels. Despite this variation, SDMA levels were not predictive of PRMT5i sensitivity. To determine whether the response of SDMA to PRMT5i was correlated with PRMT5i sensitivity, we treated cells with 10 nM PRMT5i for 4 days and analyzed SDMA levels by immunoblot. We found that SDMA levels were depleted to a similar degree across cell lines, and any variation in SDMA levels with PRMT5i treatment did not correlate with sensitivity. Therefore we conclude that neither PRMT5 nor SDMA levels could serve as a biomarker for sensitivity to PRMT5i in MCC. Mechanistically, SDMA response to PRMT5i treatment cannot explain sensitivity and additional possibilities need to be considered.

P53 status was previously linked to PRMT5i sensitivity^{74,75}, and from our sensitivity curves in Figure 3.2 p53 status was correlated with sensitivity in MCCP cells. One proposed mechanism of PRMT5 regulation of p53 is through safeguarding proper splicing of MDM4. We therefore treated a panel of MCCP cells with PRMT5i and

asked whether there was evidence for MDM4 loss and p53 activation by immunoblot (Figure 3.6A). We found that MDM4 loss was consistent across multiple cell lines, with a decrease in MDM4 levels in WaGa, PeTa, MKL-1, and MS-1 cells. We also saw elevated levels of p53 with PRMT5i treatment in all cell lines except MKL-2, as MKL-2 cells don't express any form of p53. Levels of p21, a downstream target of p53, were upregulated in WaGa, PeTa, and MKL-1 cells but not in MS-1 as these cells express a mutant form of p53 that prevents p53 gene transactivation¹⁵³.

PRMT5 has also been found to regulate p21 expression in a p53-independent manner^{154,155,156}, so we asked whether p21 activation was specific to a p53 response using p53 KO MKL-1 cell lines that have been previously described⁷³ (Figure 3.6B). We found that treating MKL-1 cells with PRMT5i for 2 days was sufficient to reduce SDMA levels but did not affect MDM4 or p21 levels. At 4 days of treatment, we saw a near complete loss of SDMA and a reduction in MDM4 levels in both lines. P21 expression was activated only in the control cell lines and not in the p53 KO line, indicating that PRMT5 loss induces p21 expression in a p53-dependent manner in MCC cells.

P53 activation can induce cell cycle arrest and apoptosis, so we next assessed cell cycle distribution with PRMT5i treatment in WaGa and MKL-1 cells (Figure 3.6C). We found that WaGa cells began accumulating in G1 after only two days of treatment with 10 nM PRMT5i. In contrast, MKL-1 cells accumulated in the G2/M phase of the cell cycle after 8 days of 10 nM PRMT5i treatment. We also asked whether the cells were undergoing apoptosis using an annexin V/propidium iodide (PI) staining assay with WaGa cells treated with PRMT5i for 4, 8, and 12 days (Figure 3.6D-E). We found that the percentage of live cells decreased while the percentage of apoptotic cells increased with treatment, reaching significance at 4 and 8 days of treatment

respectively. These findings support a model where PRMT5i induces p53 activation leading to both cell cycle arrest and apoptosis of p53 WT MCCC cells.

Previous work had shown that MDM2 inhibitors in combination with drugs that target MDM4 or CK1 α induce a greater p53 response in MCC cells than single drug treatments⁶⁷. Our work has demonstrated that PRMT5i treatment leads to loss of MDM4 and an increase in p53 activity in MCC. Therefore, we asked whether nutlin-3A, an MDM2 inhibitor, synergized with PRMT5i using cell viability assays at 4 and 8 days of treatment (Figure 3.7). We first identified suitable doses to use for 4 day and 8 day treatments using nutlin-3A or PRMT5i alone (Figure 3.7A-B) before performing combination treatments (Figure 3.7C-D). Using the Loewe additivity model to calculate synergy we found that the overall synergy scores fell mostly between 0 and 10, suggesting that the combination is additive. For WaGa cells treated for 8 days, the combination was predicted to be synergistic (overall synergy score >10). These findings indicate that combining PRMT5 inhibitors with MDM2 inhibitors may be a more effective strategy than single drug treatments.

3.4.2 PRMT5 INHIBITION INDUCES P53-INDEPENDENT CELL CYCLE ARREST AND APOPTOSIS.

Previous work in other cancer types has found that p53 KO or MDM4 overexpression reduces sensitivity to PRMT5 inhibition⁷⁵. Although we showed evidence for p53 activation with PRMT5i treatment, we asked whether p53 activation is necessary for PRMT5i sensitivity in p53 WT MCCC cells. To address this question, we used both p53 KO MKL-1 cells and WaGa cells expressing a dox-inducible p53 dominant negative mutant (p53DD). We treated these cell lines with 10 nM PRMT5i and measured cell viability after 8 days compared to the respective control cell lines (Figure 3.8A-

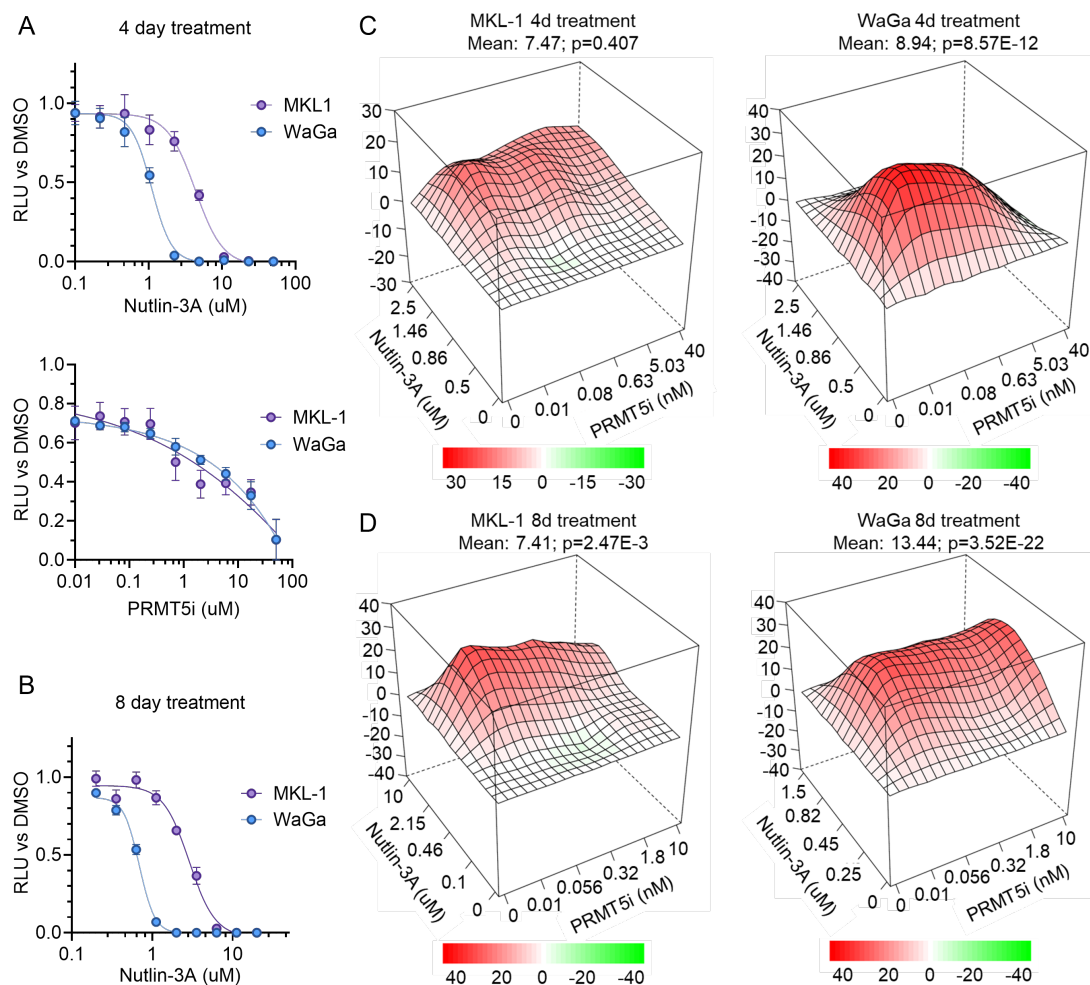


Figure 3.7: PRMT5i increases sensitivity to MDM2 inhibition. A-B. Dose response curves of MKL-1 and WaGa treated with nutlin-3A or PRMT5i inhibitor for 4 (A.) or 8 (B.) days. Cell viability was measured by CellTiter-Glo® assay. N=3. **C-D.** Synergy plots of Loewe synergy score for MKL-1 and WaGa cells treated with varying doses of both PRMT5i and nutlin-3A for 4 (C.) or 8 (D.) days. Cell viability was measured by CellTiter-Glo® assay and synergy scores were calculated using SynergyFinder Plus¹⁵⁷. N=2 for WaGa 4d treatment; N=3 for the other conditions.

D). We found that p53 loss or p53DD overexpression, as validated by immunoblot, had no effect on sensitivity to PRMT5i in either system.

Although these data clearly show that p53 was not necessary for PRMT5i sensitivity in two highly sensitive MCCP cell lines, we wanted to know whether p53 loss rescued the apoptotic or cell cycle arrest phenotypes we identified in Figure 3.6. Using the WaGa cells expressing p53DD, we found that loss of p53 activity had only a slight effect on the percentage of apoptotic cells detected with PRMT5i treatment at 4, 8, and 12 days compared to a parental control, although this effect did not reach significance (Figure 3.8E). We repeated the cell cycle flow cytometry analysis using p53DD expressing WaGa cells and found that p53DD expression had no effect on PRMT5i-mediated G1 cell cycle arrest compared with a GFP control (Figure 3.8F). By contrast, p53DD expression had a significant effect on the G1 cell cycle arrest observed with increasing doses of nutlin-3A. These findings suggest that PRMT5 loss leads to p53-independent apoptosis and cell cycle arrest.

3.5 DISCUSSION

In this chapter, we identified PRMT5 as a potential therapeutic target for MCC. We found that treatment with JNJ-64619178 (referred to here as PRMT5i) was at least 250 times more potent than GSK-3326595 in MCCP cells although they were both effective despite having different mechanisms of action. Both MCCP and MCCN cells, including two patient-derived cell lines, were sensitive to PRMT5i treatment. MCCP cell sensitivity correlated with p53 status while MCCN cells were sensitive regardless of p53 status, although this conclusion is somewhat limited by the number of cell lines we were able to test. We tested seven MCCP cell lines, two of which were p53

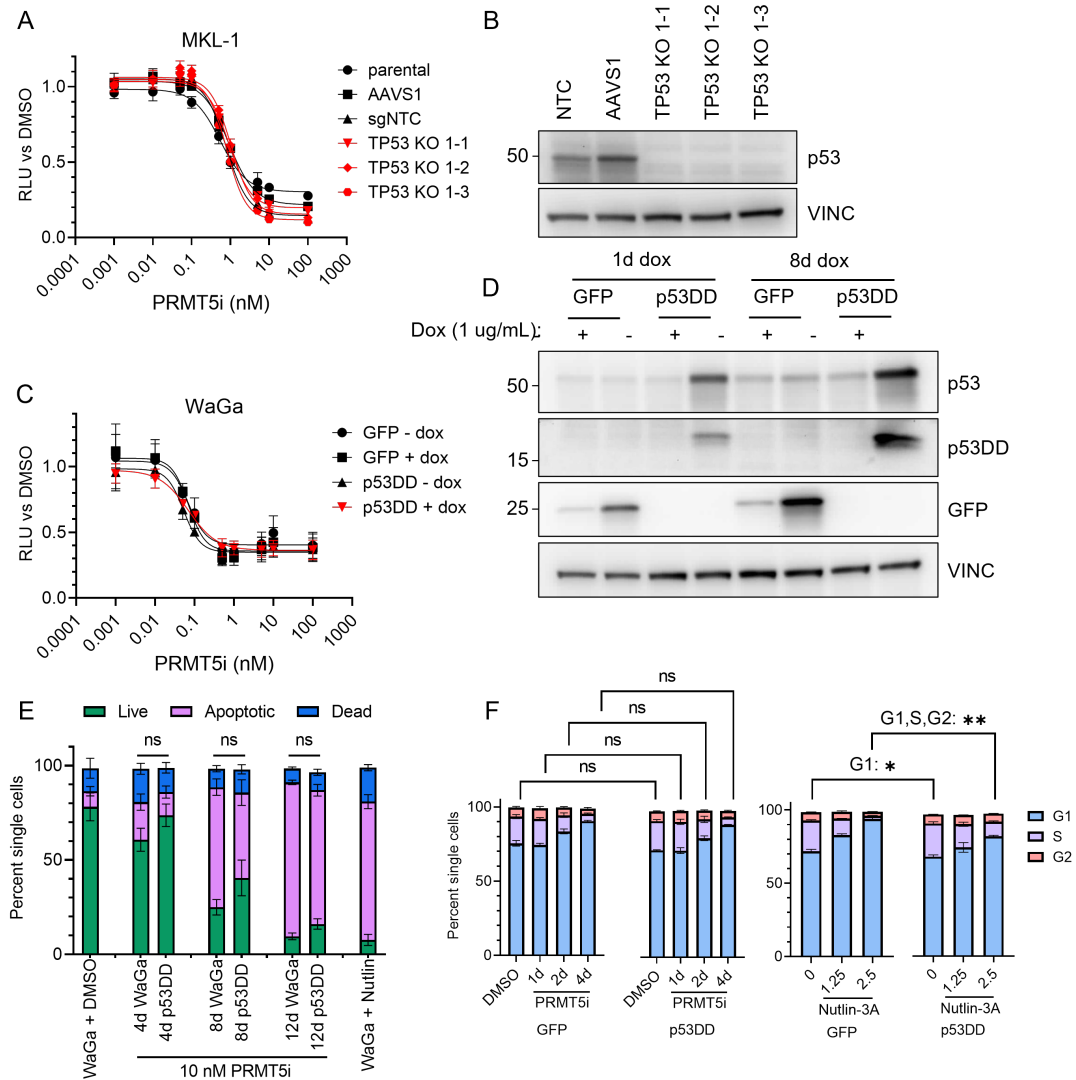


Figure 3.8: p53 is not required for sensitivity to PRMT5i. **A.** Cell viability measured by CellTiter-Glo® of p53 KO or control MKL-1 cell lines from Ananthapadmanabhan et al.⁷³ NTC = non targeting control; AAVS1 = Adeno-associated virus integration site 1. N=3. **B.** Immunoblot of MKL-1 cell lines from A. **C.** Cell viability via CellTiter-Glo® of WaGa cells expressing doxycycline (dox)-inducible GFP or p53DD, a p53 dominant negative mutant. N=3. **D.** Immunoblot of WaGa cell lines from C. treated with 1 ug/mL dox for 1 or 8 days. **E.** Summary plot of Annexin V-FITC/PI staining of parental WaGa cells compared with p53DD-expressing WaGa cells. N=3. **F.** Summary plots of cell cycle distribution flow experiments of WaGa cells expressing GFP or p53DD treated with either 10 nM PRMT5i (left) for the indicated time or with nutlin-3A (right) for 1 day at the indicated dose. N=3.

mutant, and four MCCN cell lines, three of which were p53 mutant. Previous work investigating 25 different cancer cell lines found that p53 status was not correlated with PRMT5 inhibitor sensitivity⁹⁹, while an additional study testing over 200 cell lines came to the opposite conclusion⁷⁵, highlighting the importance of sample size in correlative studies. Testing additional MCC cell lines, particularly those from p53 mutant MCCP and p53 WT MCCN tumors, would be useful to determine whether p53 status is correlated with PRMT5i sensitivity.

The dose and length of treatment are important considerations when investigating the molecular effects of an inhibitor. In the case of PRMT5 inhibition, previous work with JNJ-64619178¹⁵² and GSK-3203591⁷⁵ (closely related to GSK-3326595) treated cells for 6-10 days before measuring cell viability, whereas work using GSK-3235025 (also known as EPZ-015666, a precursor to GSK-3326595) treated cells up to 12 days. We therefore assessed the effect of both dose and time on MCC cell growth over a total of 12 days and found that there was a dose effect of PRMT5i on cell count at doses up to 5 nM, at which point the length of treatment had a greater effect on cell growth. Cell counts continued to increase between 0 and 4 days even at the highest doses tested. At doses >5 nM, the cell counts remain somewhat stable between 4 and 8 days before decreasing between 8 and 12 days. Based on this experiment, we chose 10 nM for most of our subsequent assays. We chose 10 nM over 5 nM for two reasons: one, the difference in cell count between DMSO and PRMT5i treatment after 4 days reached significance with 10 nM; and two, because WaGa cells are the most sensitive MCCP cell line we tested and we wanted choose a dose that would be effective in our less sensitive lines as well.

To investigate the mechanisms contributing to PRMT5i sensitivity, we first showed that neither PRMT5 levels nor response to PRMT5i as measured by SDMA loss was

predictive of response. PRMT5 levels were very consistent across MCC cell lines, even more consistent than proteins typically used as immunoblot loading controls like vinculin (data not shown) or TBP suggesting that PRMT5 levels are not indicative of sensitivity. An interesting question not examined here is how PRMT5 and SDMA levels in MCC compare with levels in normal Merkel cells, adjacent skin cells, or even other PRMT5i sensitive cancers. It's possible that PRMT5 levels are elevated in the cancerous cells compared with normal tissue, and it would be interesting to see how PRMT5 levels in MCC compare with other PRMT5i-sensitive cancers.

P53 is a well described tumor suppressor that has been previously shown to be activated following loss of PRMT5, and loss of p53 has been shown to attenuate PRMT5 inhibitor sensitivity in a number of cell lines^{75,147,110,77,74,76}. We found that MDM4, a negative regulator of p53, decreased with PRMT5i treatment while p53 levels increased for most MCCP cells. P53 appeared to be activated by PRM5i treatment as p21 levels increased in a p53-dependent manner. We also further characterized the response to PRMT5i and found that PRMT5i treatment induced cell cycle arrest and apoptosis as measured by annexin V staining. However, knockout of p53 or overexpression of a dominant negative p53 mutant (p53DD) had no effect on MCCP sensitivity to PRMT5i. There was also no significant change in the cell cycle distribution or percentage of apoptotic cells with PRMT5i treatment in a p53DD-expressing cell line compared to a parental control, suggesting that PRMT5 induces a p53-independent apoptotic response. In the next chapter, we investigate alternative functions of PRMT5 in an effort to identify p53-independent molecular pathways that contribute to PRMT5i sensitivity.

AUTHOR CONTRIBUTIONS FOR CHAPTER 4

The lentivirus for the CRISPR screen was generated and titered by Thomas C. Frost and Ashley K. Gartin. The alternative splicing analysis of the RNA sequencing data was performed by Colin E. Fowler. The quantitative proteomics experiment was carried out by Joao A. Paulo with sample preparation performed by Camille H. Cushman. Differential protein analysis was performed by Ziad El Bakouny. All other experiments were performed by Camille H. Cushman with input from James A. DeCaprio.

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4

Identifying and characterizing mRNA processing as a critical feature of PRMT5 inhibitor sensitivity in MCC.

4.1 ABSTRACT

PRMT5 is an arginine methyltransferase that has roles in regulating gene expression, mRNA splicing, DNA damage repair, among many others. In Chapter 3, we

found that many Merkel cell carcinoma cell lines were sensitive to PRMT5 inhibition and showed that this was independent of p53 activity. Here, using a CRISPR screen we identified mRNA processing as a critical feature of the oncogenic activities of PRMT5. We characterized the effect of PRMT5 on mRNA splicing and find that PRMT5 regulates removal of detained introns (DIs) from a subset of genes. We showed that loss of PRMT5 affected removal of DIs from genes involved in DNA damage repair and cell cycle progression. To investigate the effect of PRMT5 splicing defects on global protein expression, we performed quantitative mass spectrometry experiments of MCC cells treated with a PRMT5 inhibitor. We showed that PRMT5 loss induces widespread increases and decreases in protein expression and found that shortly after PRMT5 inhibition the cell upregulates expression of proteins involved in mRNA processing, many of which were identified in the CRISPR screen. We found that changes in mRNA expression with PRMT5 inhibitor treatment weakly correlated with protein expression and that correcting for increased levels of DIs improve this correlation for specific gene sets. We propose a model where PRMT5 loss induces defects in mRNA splicing of DIs leading to an upregulation of additional mRNA processing factors in an attempt to compensate for loss of this critical regulatory pathway.

4.2 INTRODUCTION

In Chapter 3, we found that PRMT5 inhibitor treatment was toxic to many MCC cell lines independently of cellular PRMT5 levels, SDMA loss, or p53 activity. Reading the peer-reviewed literature on PRMT5 function, it's easy to find numerous alternative PRMT5 targets whose methylation may be required for MCC viability such as: transcription factors like E2F1 ^{112,158,159}, KLF4 ¹⁶⁰, or FOXP3 ¹⁶¹; histone tar-

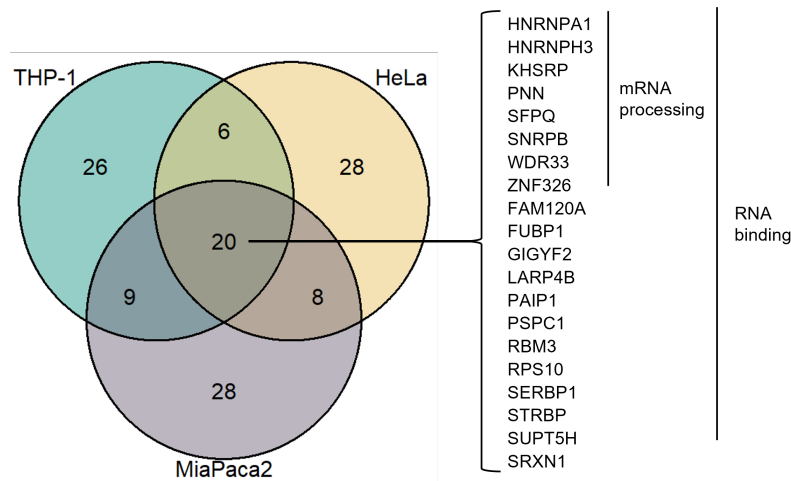


Figure 4.1: Previously identified PRMT5 targets between cell types are enriched for RNA binding and mRNA processing factors. Venn diagram of PRMT5 targets identified from Radzisheuskaya et al.⁸⁹ (THP-1), Musiani et al.⁹⁰ (HeLa), and Mulvaney et al.⁸³ (MiaPaca2). Targets shared between all three studies are listed on the right. Gene ontology analysis using DAVID found a significant enrichment of genes involved in RNA binding and mRNA processing ($q < 0.05$).

gets such as H3R2, H3R8, and H4R3^{162,163,103,104}; DNA damage proteins like RAD9 or RUVBL1^{116,164}; and mRNA processing factors like Sm B/B' (SNRPB)^{97,165} or SRSF1⁸⁹ (reviewed in⁷⁹). At least three studies have used mass spectrometry-based approaches to identify PRMT5 methylation targets in different cell lines^{83,90,89}. Each of these studies enriched for symmetrically dimethylated arginine (SDMA) residues by immunoprecipitation before analyzing proteins by mass spectrometry. Combining the methylation targets from these studies, we see that the shared targets between cell lines are almost entirely RNA-binding proteins, many of which have previously been implicated in mRNA processing (Figure 4.1). We refer to these genes as “high-confidence” PRMT5 targets as they were independently identified in three different systems. It’s possible that they are either the most abundantly methylated or most highly conserved PRMT5 targets between cell types.

Many studies in addition to these three have found that PRMT5 can regulate mRNA processing through modulating the activity of mRNA splicing factors and spliceosome components. The major spliceosome is a highly complex molecular machine, composed of five small nuclear RNAs (snRNAs) known as U1, U2, U4, U5, and U6, and about 100 proteins (reviewed in ^{166,167,168}). The snRNAs contain U-rich sites at the 3' end known as the Sm site where a ring of seven Sm proteins bind. These proteins are Sm B/B' (a splice variant of Sm B), Sm D1, Sm D2, SmD3, Sm E, and Sm F. In the case of U6, the snRNA is instead threaded through a ring of seven paralogous LSm ("Like"-Sm) proteins, LSm2-8. SnRNAs bound to either the Sm or LSm ring along with a unique set of additional proteins are called small nuclear ribonuclear proteins, or snRNPs, and are responsible for catalyzing each step in the splicing reaction.

Assembly of the Sm core around each of the snRNAs is mediated through binding to the SMN complex, a complex of proteins including SMN, unrip, and Geminin2-8 (reviewed in ^{167,168}; Figure 4.2). PRMT5, in complex with WDR77 (also known as MEP50 or WD45) and an adaptor protein known as pICln (*CLNS1A*), is thought to methylate the RG-rich RNA-binding motifs of the Sm proteins B/B', D1, and D3 to increase affinity for the Tudor domain of the SMN protein. The SMN complex facilitates binding of the Sm proteins around the snRNA at the Sm site and transports with the snRNA into the nucleus. The snRNA undergoes additional modification in subnuclear domains known as Cajal bodies before dissociating from SMN and binding with additional snRNA-specific spliceosomal proteins to form snRNPs. The SMN complex exits the nucleus into the cytoplasm where it can re-enter the snRNP biogenesis pathway.

In addition to methylation of core spliceosomal proteins, PRMT5 has been implicated in methylation of a number of splicing factors like SRSF1 ⁸⁹. Loss of PRMT5

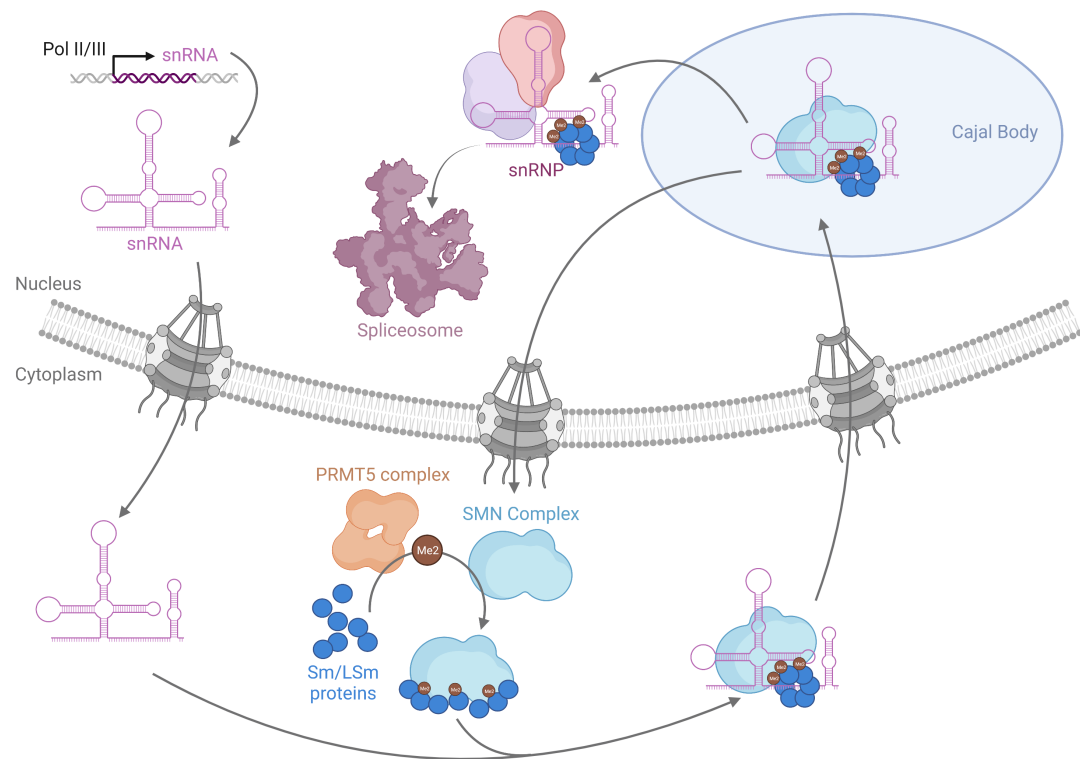


Figure 4.2: Assembly of spliceosomal snRNPs. Cartoon of snRNP assembly adapted from¹⁶⁸. Created with BioRender.com.

leads to splicing dysregulation, particularly leading to an increase in detained introns and skipped exons at weak splice sites^{74,99,75,169}. The degree to which PRMT5 affects specific alternative splicing events varies between studies: Braun et al.⁹⁹ found that PRMT5 loss leads predominantly to an increase in detained introns while Gerhart et al.⁷⁵ found that the greatest change in splicing variation with PRMT5 loss was skipped exons.

In this chapter, we performed three genome-wide assays to identify PRMT5i vulnerabilities. We performed a CRISPR screen and found that loss of mRNA processing factors increases MCCP sensitivity to PRMT5 inhibition. We characterized the effect of PRMT5 on mRNA splicing by RNA-seq and determine that PRMT5 loss predominantly leads to an increase in detained introns of cell cycle regulatory and DNA damage genes. We performed quantitative mass spectrometry to measure changes in global protein expression with PRMT5 inhibition and found that mRNA processing factors increased in expression with PRMT5i treatment. We analyzed the correlation between mRNA and protein expression changes and found low correlation coefficients, even when correcting for DIs. Overall, we conclude that PRMT5-regulated splicing of detained introns is essential in MCC but that identifying splicing changes in mRNA expression is not sufficient to predict protein expression changes.

4.3 METHODS

4.3.1 CELL CULTURE

MCC cell lines were gifts from Masahiro Shuda (University of Pittsburgh), Jürgen Becker (Translational Skin Cancer Research, German Cancer Consortium (DKTK), Essen, Germany), and Roland Houben (University of Wuerzburg, Germany). Cells

were cultured in RPMI (Life Technologies, 11875119) with 10% FBS and 1% penicillin-streptomycin (Life Technologies 15140163). Cell lines were confirmed to be free of mycoplasma contamination using the e-Myco Mycoplasma PCR Detection Kit (Bulldog Bio 25233).

4.3.2 H3 CRISPR SCREEN

The H3 CRISPR-Cas9 protocol for suspension cells (<https://www.addgene.org/pooled-library/liu-crispr-knockout-h3/>) was followed with a few modifications. Briefly, MKL-1 cells were dissociated with accutase (StemCell Technologies 07920) prior to spinfection of 1×10^6 cells/mL with lentiviral supernatant encoding the H3 library, sufficient for 30% survival after puromycin selection. Spinfection was performed as described in the Broad Optimization of lentiviral transduction using spinfection protocol (https://portals.broadinstitute.org/gpp/public/dir/download?dirpath=protocols/production&filename=Optimization_of_Lentiviral_Spinfection_Oct2018.pdf). After selection, 5×10^7 accutase-treated cells were harvested and the remainder was plated at 5×10^5 cells/mL in either 0.2% DMSO or 0.15 nM PRMT5i (JNJ-64619178). Every 3 days, cells were accutased, counted, and replated at 5×10^5 cells/mL. Cells were harvested after 15 days of treatment. DNA was isolated via phenol/chloroform extraction and the library was amplified for sequencing according to the Addgene protocol.

DATA ANALYSIS:

High-throughput sequencing was performed by Novogene Corporation Inc. using the NovaSeq 6000. >30 million PE150 reads were obtained and sequence quality was assessed by FastQC, however only read 1 was used for analysis as recommended by

the Addgene protocol. Identification of the differential abundance of guides in each sample was performed using the MAGeCKFlute pipeline¹⁷⁰, specifically using the MAGeCK-VISPR algorithm¹⁷¹ (MLE) to calculate beta scores with cell cycle normalization.

4.3.3 RNA-SEQUENCING

MKL-1 and WaGa cells were treated with 10 nM PRMT5i for 4 or 8 days prior to harvesting. Total RNA was extracted using TRIzolTM (Life Technologies 15596026) according to manufacturer's recommendation. Poly A selection, library preparation, and sequencing was performed by Novogene Corporation Inc. Sequencing was performed on the NovaSeq 6000 to >12G (40 million reads) per sample of PE150 reads.

DATA ANALYSIS:

TOTAL RNA EXPRESSION: Sequence quality was assessed with FastQC (Babraham Bioinformatics) and read alignment to the GRCH38p.13 v101 cDNA library was performed using Salmon¹⁷². Differential expression analysis was performed using DESeq2^{173,174}. Gene ontology analyses were restricted to genes with a HUGO gene symbol and performed using DAVID¹⁷⁵.

ALTERNATIVE SPLICING ANALYSIS: Raw RNA-seq reads were mapped using STAR aligner version 2.5.3a¹⁷⁶ with the parameters:

```
STAR --runMode alignReads
      --runThreadN 16
      --sjdbOverhang 149
      --outReadsUnmapped Fastx
```

```

--outSAMtype BAM SortedByCoordinate
--outFilterMultimapNmax 20
--outFilterMismatchNmax 999
--outFilterMismatchNoverLmax 0.04
--alignIntronMin 70
--alignIntronMax 500000
--alignMatesGapMax 500000
--alignSJoverhangMin 8
--alignSJDBoverhangMin 1
--outSAMstrandField intronMotif
--outFilterType BySJout
--twopassMode Basic

```

Additionally, raw RNA-seq reads were mapped using Bowtie 1.2.3¹⁷⁷ with the parameters:

```
bowtie -n 1 -l 30 -e 70 -t -p 4 -m 1 -X 500 -S
```

Identification and Quantitation of Detained Introns and Other Alternative Splicing Events: Detained intron and alternative splicing event identification was performed as described in Boutz et al. 2015¹¹⁴ and Braun et al. 2017⁹⁹, with the following modifications: Mapped reads were filtered using Bedtools 2.26.0¹⁷⁸ to remove reads overlapping expressed repeats from the UCSC genome browser RNA repeats and Gencode hg38 and coding exons annotated in the Gencode hg38 annotations. Alternative and constitutive classifications were performed using custom Python scripts, and are agnostic with regard to existing annotations other than known gene boundaries. Importantly, both WaGa and MKL-1 cell lines were run in parallel to ensure that the annota-

tions would be consistent between the cell lines.

Detained Intron and Alternative Splicing Event Quantification: Detained intron and alternative splicing events were quantified using DEXSeq¹⁷⁹. A genome annotation including all alternative splicing events and detained introns was generated using custom python scripts and python scripts that accompany DEXSeq. Reads were then quantified to this genome using the DEXSeq python script with the following parameters:

```
python dexseq\_count.py -p yes -s no -f bam -r pos
```

Finally, differential splicing was quantified using the DEXSeq package within R. Alternative splicing events and detained introns were called as significant if the FDR-corrected p-value < 0.05.

Genome-Wide Transcript Annotation: Transcript annotation was performed as described in Braun et al., 2017⁹⁹. Briefly, we derived a complete transcriptome annotation based on Gencode hg38 gene start and end boundaries and the location of polyA sites for data derived from the WaGa and MKL-1 cell lines, the genomic locations of detained introns as determined above, and the genomic locations of all splice junctions using custom Python scripts. For comparisons of isoforms containing detained introns to the consensus coding sequence, the annotation for each gene was set to identify the consensus isoform, i.e. the junctions defining each exon are the most frequently used junction detected within all of the combined samples. Each detained intron within a gene was then assigned to an additional transcript.

Gene and Isoform Expression: Gene-level and isoform-level quantification was performed using Rsem version 1.3.1¹⁸⁰ and EBSeq¹⁸¹ using the custom genomic annotation described above, as well as Gencode hg38 annotations with all three biological

replicates each for DMSO control and PRMT5i-treated cells with the parameters:

```
rsem-calculate-expression --forward-prob 0.5  
--no-bam-output --paired-end --p 4
```

followed by read-count matrix assembly and standard EBSeq differential expression analysis. Gene and isoform expression differences at the total gene level were considered significant if the posterior probability of differential expression (PPDE) as determined by EBSeq was > 0.95 .

Other software used:

Python 2.7.13

R 3.5.1 and 3.6.0

Perl 5.24.1

Picard 2.18.26

4.3.4 QUANTITATIVE PROTEOMICS

Quantitative proteomics was performed as previously described^{182,183} based on the SL-TMT mass spectrometry protocol¹⁸⁴. Briefly, cells were treated with 10 nM PRMT5i for 4, 8, or 12 days prior to harvesting. Cells were lysed in lysis buffer (8M urea, 200 mM EPPS pH 8.5, and protease inhibitors (Pierce, A32953)) and homogenized by passing through a 20G needle. Protein was quantified by BCA followed by disulfide bond reduction with 5 mM Pierce neutralized TCEP for 15 min at room temperature protected from light. Alkylation with 10 mM iodoacetamide was performed by incubating at room temperature for 30 minutes protected from light before the reaction was quenched with 5 mM DTT. 100 ug of protein was collected for chloroform-methanol precipitation and resuspended in EPPS buffer (200 mM, pH 8.5). Samples

were digested at a 1:100 enzyme-to-protein ratio with LysC protease at room temperature overnight. Samples were then digested again at a 1:100 enzyme-to-protein with trypsin for 6 hours at 37° C.

Approximately 50 ug of peptides from each sample was labelled with 100 ug TMT reagent which were dissolved in anhydrous ACN to achieve a final concentration of 30% (v/v). TMT-labelled samples were acidified, vacuum centrifuged to near dryness and subjected to C18 SPE (Sep-Pak, Waters). Samples were subjected to basic pH reversed-phase HPLC. Offline fractionation was performed using an Agilent 1200 pump with an Agilent 300Extend C18 column (3.5 μ m particles, 2.1 mm ID and 250 mm in length). Peptides were subjected to a 50 min linear gradient from 5% to 35% acetonitrile in 10 mM ammonium bicarbonate pH 8 at a column flow rate of 0.25 mL/min. Ninety-six fractions were collected and then concatenated into 24, of which 12 non-adjacent samples were analyzed.

Data were obtained with Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific) coupled with a Proxeon EASY-nLC 1200 LC pump (Thermo Fisher Scientific). Peptide separation was done using a custom Accucore C18 resin (2.6 μ m, 100A, ThermoFisher Scientific) column for 3 hrs using a gradient of 6-30% ACN in 0.125% formic acid with a flow rate of 300 nL/min. The Thermo FAIMS Pro interface was operated with default parameters (the inner and outer electrode were set at 100°C, the dispersion voltage (DV) was set at -5000 V, and no additional gas was used). One CV set included -40V, -60V, and -80V. All analysis involved an hr-MS2-based TMT method as previously mentioned¹⁸⁴ and mass spectra was processed as previously described¹⁸².

Raw files were first converted to mzXML. Database searching included all human entries from UniProt. The database was concatenated with one composed of all pro-

tein sequences in the reversed order. Sequences of common contaminant proteins were also included. Searches were performed using a 50-ppm precursor ion tolerance and 0.03Da product ion tolerance. TMTpro on lysine residues and peptide N termini (+304.2071Da) and carbamidomethylation of cysteine residues (+57.0215Da) were set as static modifications (except when testing for labeling efficiency, when the TMTpro modifications were set to variable), while oxidation of methionine residues (+15.9949Da) was set as a variable modification.

Peptide spectrum matches (PSMs) were adjusted to a 1% false discovery rate (FDR)¹⁸⁵. PSM filtering was performed using linear discriminant analysis (LDA) as described previously¹⁸⁶, while considering the following parameters: XCorr, Cn, missed cleavages, peptide length, charge state, and precursor mass accuracy. Protein-level FDR was subsequently set at 1%. For reporter ion quantification, a 0.003Da window around the theoretical m/z of each reporter ion was scanned, and the most intense m/z was used. Peptides were filtered to include only those with a summed signal-to-noise ratio 100 across all channels. For each protein, the filtered signal-to-noise values were summed to generate protein quantification values. To control for different total protein loading within an experiment, the summed protein quantities of each channel were adjusted to be equal in the experiment.

DIFFERENTIAL PROTEIN EXPRESSION ANALYSIS

Protein levels were batch corrected using ComBat¹⁸⁷. Linear modeling of changes in protein levels over time was performed in R (R version 4.2.0) using the R stats package with protein expression as the dependent variable and time as the independent variable for each protein. Linear model coefficients were calculated as the slope 'a' in the linear equation 'y= ax + b'. P values were adjusted for multiple hypothesis cor-

rection using the Benjamini-Hochberg procedure and statistical significance was determined as an adjusted p value < 0.05 . Gene ontology analysis was performed using DAVID with total detected proteins as background. UniProt (UP) and gene ontology (GO) biological process and molecular function terms were included in the analysis.

4.4 RESULTS

4.4.1 PRMT5 INHIBITOR TREATMENT INCREASES DEPENDENCE ON FACTORS INVOLVED IN MRNA PROCESSING.

The breadth of previously described PRMT5 targets makes it challenging to determine which functions of PRMT5 are most critical to MCC growth. In the previous chapter, we showed that p53 is activated with PRMT5 loss but is not required for PRMT5i sensitivity indicating that there are additional mechanisms of PRMT5 dependence in MCC. To identify pathways involved in PRMT5 sensitivity in MCC, we performed a CRISPR screen in MKL-1 cells treated with PRMT5i (Figure 4.3A). We used the H3 human CRISPR knockout library developed by Xiaole Shirley Liu and Myles Brown (Addgene pooled library #133914) targeting more than 18,000 genes with 6 guides per gene computationally-predicted and experimentally-observed to have maximum knockout efficiency. We calculated the IC₃₀ of MKL-1 cells treated with PRMT5i for 15 days to be close to 0.15 nM (Figure 4.3B). We collected samples for library preparation and sequencing immediately after library selection (Day 0) and after 15 days of DMSO or PRMT5i treatment to identify both positively- and negatively-selected genes. Using the MAGeCKFlute analysis pipeline¹⁷⁰ we calculated the difference in cell cycle-normalized beta scores between DMSO treated and PRMT5i treated samples and ranked these values by intensity, highlighting the hits that had a difference in

beta scores greater than two standard deviations ($SD > 2$) (Figure 4.3C). WDR77, the cofactor of PRMT5, was negatively selected which aligns with our understanding of PRMT5 activity. Notably, *TP53* was not strongly positively (or negatively) selected in our PRMT5i treated samples in agreement with our work from Chapter 3. A complete list of negatively and positively selected hits ($SD > 2$) can be found in the appendix in Table A.1 and Table A.2 respectively.

To identify molecular pathways that contribute to PRMT5 sensitivity or resistance, we performed a gene ontology enrichment analysis using DAVID^{188,175} (Figure 4.3D). Among the positively selected genes, ribosomal proteins were significantly enriched including components of both the 60S and 40S ribosomal subunits. PRMT5 has previously been linked to regulating ribosomal assembly and protein translation^{87,93} although none of the ribosomal proteins previously reported to be methylated by PRMT5 were identified in our screen. Among the negatively selected genes, genes involved in mRNA processing and RNA binding were significantly enriched. These terms mirror those enriched among the high-confidence PRMT5 targets and in fact 3 out of the 20 high-confidence PRMT5 targets were also negatively selected hits in the CRISPR screen, specifically PAIP1, SERBP1, and SUPT5H. PAIP1 is involved in mRNA circularization to promote translation initiation¹⁸⁹; SERBP1 has been implicated in regulating in ribosome activity⁹⁴ and methionine production¹⁹⁰; and SUPT5H is a transcription elongation factor reportedly involved in mRNA capping¹⁹¹.

We also examined how the known PRMT5 adaptor proteins CLNS1A, COPR5, and RIOK1 scored in our screen. We found that COPR5 (*COPRS* gene) was positively selected with a $SD > 2$. Although the difference in beta scores for both RIOK1 and CLNS1A did not reach the $SD > 2$ threshold, we saw that RIOK1 had a positive difference in beta scores (PRMT5i-DMSO Beta score = 0.65) while CLNS1A had a

negative difference in scores (PRMT5i-DMSO Beta score = -0.56). CLNS1A promotes PRMT5 methylation of mRNA splicing and processing factors while RIOK1 and COPR5 promote methylation of ribosomal proteins and histones respectively. This finding, in combination with the enrichment of mRNA splicing factors among the negatively selected genes, suggests that the role of PRMT5 in splicing regulation is a key contributor to PRMT5 inhibitor sensitivity in MCC.

4.4.2 PRMT5 IS REQUIRED FOR SPLICING OF DETAINED INTRONS FROM GENES INVOLVED IN THE CELL CYCLE AND DNA DAMAGE RESPONSE.

Based on the findings from our CRISPR screen, we decided to characterize the effect of PRMT5 inhibition on mRNA expression and splicing in MCC. We performed high throughput RNA sequencing of MKL-1 and WaGa cells treated with 10 nM PRMT5i for 4 and 8 days. We first analyzed the effect of PRMT5i treatment on total RNA expression over time. We performed a principal component analysis of the expression data and found that most of the variance can be explained along one principal component, and that the 4 day and 8 day treated samples cluster close together along the first component, noting that there was more variation in the MKL-1 data (Figure 4.4A).

We compared the effect of the 4 day PRMT5i treatment to the 8 day treatment by correlating the log-transformed fold change in PRMT5i-treated gene expression vs DMSO and found for both WaGa and MKL-1 cells there was a highly significant correlation between time points with a linear regression equation very close to $y=x$. (Figure 4.4B). These findings suggest that the greatest effect of PRMT5 inhibition occurs within 4 days of PRMT5i treatment and that these changes are sustained up to 8 days of treatment.

We decided therefore to focus our efforts on characterizing the effects of PRMT5i

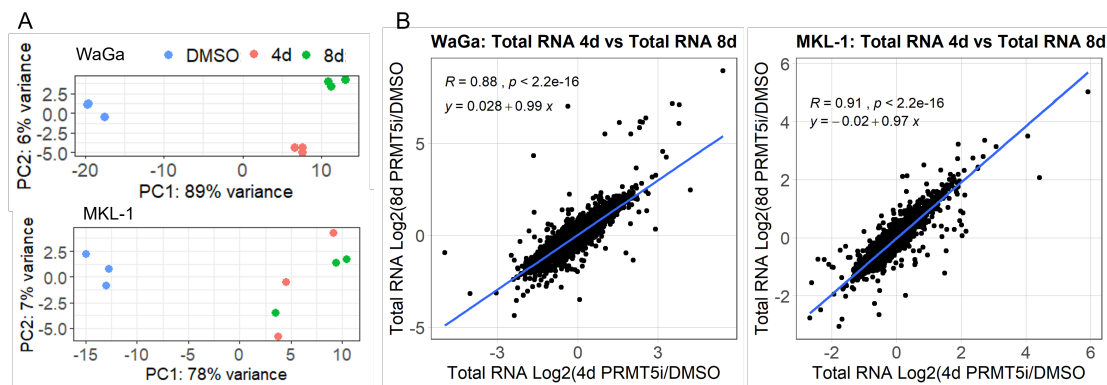


Figure 4.4: Most effects of PRMT5i on total RNA expression occur within 4 days and persist to 8 days of treatment. **A.** Principal component analysis of total RNA expression data transformed with the variance stabilizing transformation from WaGa and MKL-1 cells in triplicate. **B.** Correlation between log-transformed fold change of expression of PRMT5i-treated samples vs DMSO at 4 and 8 days of treatment from WaGa (left) and MKL-1 (right).

on mRNA expression after 4 days of treatment. We identified the top positively and negatively selected genes ($p_{adj} < 0.05$; $|\log_2FC| > 1$) in MKL-1 and WaGa cells and performed a gene ontology analysis using DAVID (Figure 4.5A-B). Although there were many significantly upregulated genes with PRMT5i treatment, there was no specific biological process or molecular function GO term enrichment (data not shown). We did see that genes involved in cell division and mitosis were significantly enriched among the genes downregulated in WaGa cells, but not in MKL-1 cells. These included key cell cycle regulatory genes associated with progression through mitosis such as CCNB1, CCNB2, CDK1, and AURKA. These findings are in alignment with the WaGa-specific G1 cell cycle arrest we found in Chapter 3, Figure 3.6C. We showed that WaGa cells begin to accumulate in G1 after 2 days of treatment, so although it is theoretically possible that PRMT5 directly regulates transcription of mitotic gene expression, the loss of mitotic mRNA expression after 4 days of treatment may be an indirect consequence of this cell cycle arrest. We compared the dif-

ferentially expressed genes between WaGa and MKL-1 cells with PRMT5i treatment and found a small overlap in genes downregulated with PRMT5i treatment (Figure 4.5C). We saw a greater similarity between differentially upregulated genes in MKL-1 and WaGa cells, but again found no specific GO terms enriched in the shared gene set (data not shown).

Because we performed deep sequencing of our samples up to 40 million reads per sample, we were able to measure the effect of PRMT5i treatment on alternative splicing events in MCC. We first examined how PRMT5i treatment affected splicing of MDM4 (Figure 4.6). Previous work found that PRMT5 inhibition can lead to increased skipping of exon 6 and increased inclusion of a detained intron (DI) directly downstream of exon 6^{74,114} (Figure 4.6A). Full-length MDM4 transcripts include all 11 canonical exons (MDM4-FL) and skipping of exon 6 leads to either nonsense-mediated decay of the transcript or the production of an unstable protein (MDM4-S) (reviewed in¹⁹²). Transcripts containing DIs are thought to remain in the nucleus and therefore do not produce any protein¹¹⁴ (MDM4-DI). In our data, we observed that exon 6 usage decreases with PRMT5i treatment while inclusion of the DI immediately downstream of exon 6 increases with PRMT5i treatment in WaGa cells (Figure 4.6B). In MKL-1 cells, we saw similar results although the increase in the inclusion of the DI did not reach significance (data not shown). These findings are in alignment with the previous literature and this change in MDM4 splicing may contribute to the loss of MDM4 expression we observed in Chapter 3 with PRMT5i treatment.

We next measured changes in global alternative splicing patterns to determine how PRMT5i treatment affects splicing patterns more broadly. We aligned reads to the whole genome and quantified differential exon usage using the DEXseq R package^{179,193} (Figure 4.7A). Specifically, we were able to quantify changes in usage of alternative

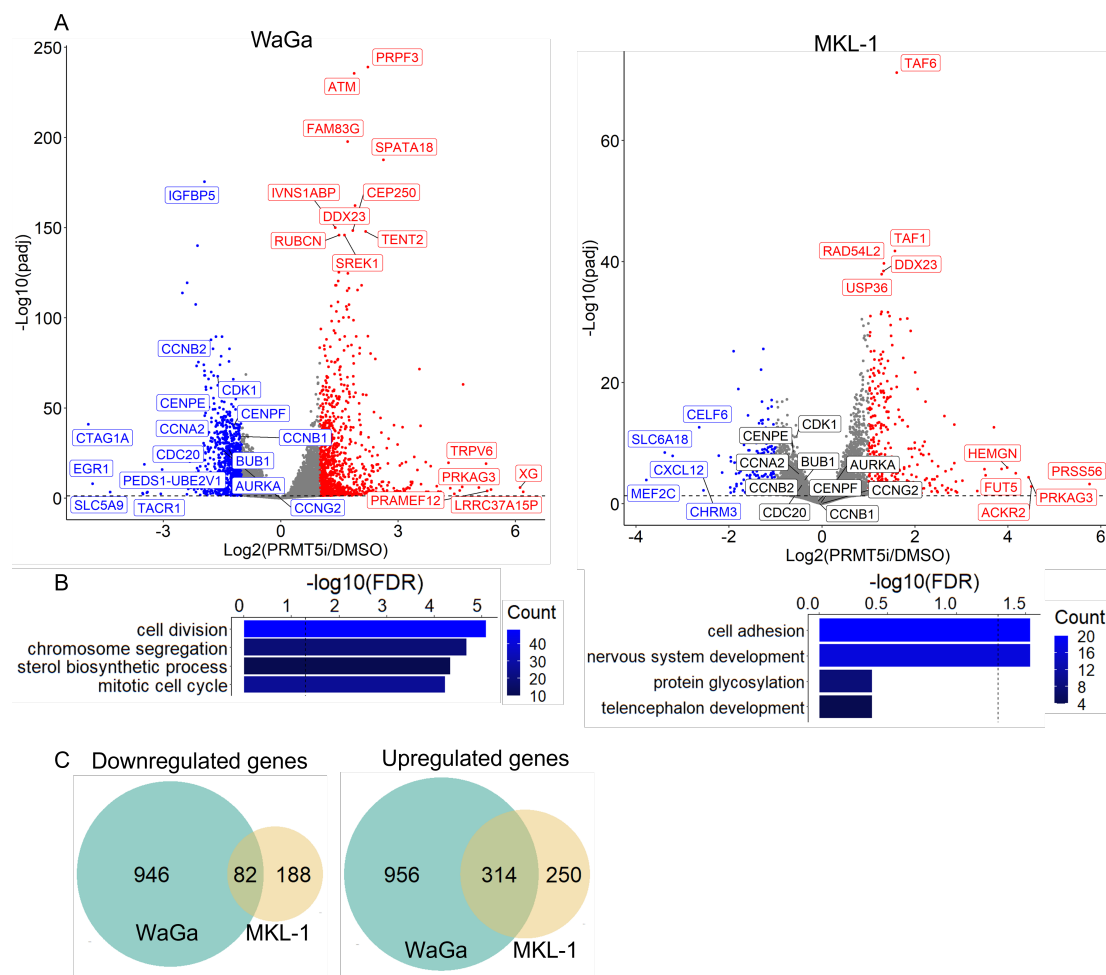


Figure 4.5: Effect of PRMT5i treatment on total gene expression is influenced by cell context. A. Volcano plots of gene expression changes in WaGa (left) and MKL-1 (right) cells treated with 10 nM PRMT5i for 4 days compared to DMSO. Significantly downregulated genes ($p_{adj} < 0.05$, $PRMT5i/DMSO < -2$) are in blue and significantly upregulated genes ($p_{adj} < 0.05$, $PRMT5i/DMSO > 2$) are in red. **B.** DAVID gene ontology enrichment analysis of downregulated genes with 4 days PRMT5i treatment vs DMSO. **C.** Overlap of significantly downregulated (top) and upregulated (bottom) genes between WaGa and MKL-1 cells treated with PRMT5i for 4 days.

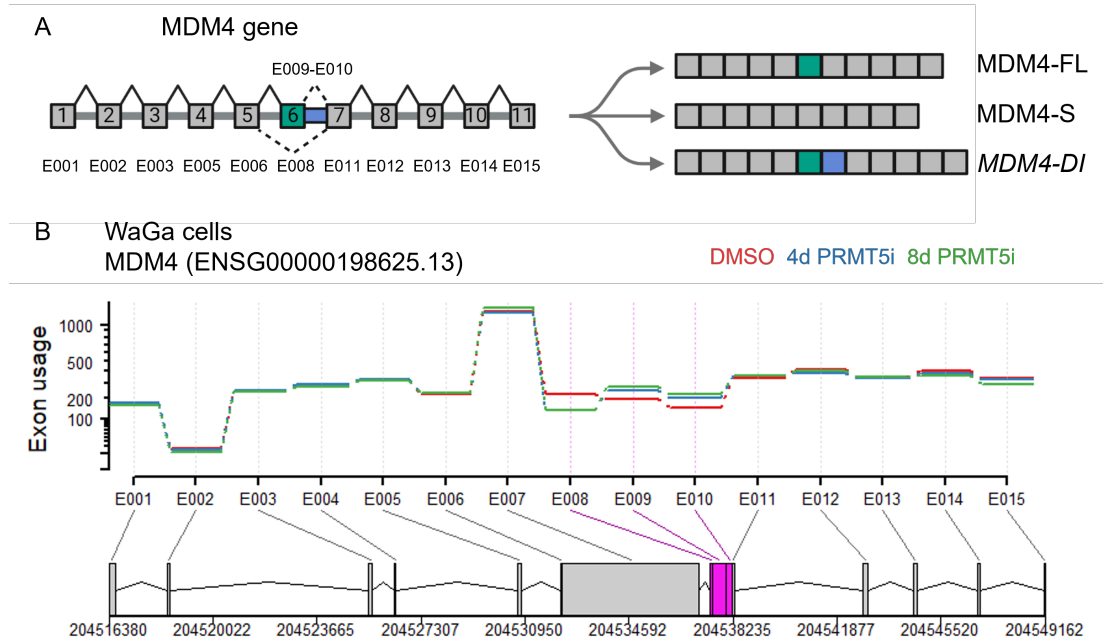


Figure 4.6: Splicing of MDM4 exon 6 and adjacent detained intron is altered with PRMT5i treatment. **A.** Diagram of the MDM4 gene and the predicted alternatively spliced transcripts affected by PRMT5 inhibition. Canonical exon number is labeled within each exon and the alternatively spliced exon is in green while the previously identified detained intron is in blue. The sequence IDs in B. are indicated below the diagram for the exons and above the diagram for the detained intron. MDM4-FL and MDM4-S can be translated whereas MDM4-DI is predicted to remain in the nucleus. **B.** Counts of exons identified in WaGa cells treated with DMSO or PRMT5i for 4 or 8 days normalized by total reads within each sample. Relative positions of each exon along chromosome 1 is indicated below the diagram.

3' splice sites (A3PSS), alternative 5' splice sites (A5PSS), cassette exons (CAS), detained introns (DI) and mutually exclusive exons (MXE). We identified total alternative splice events (Total AS) detected in either DMSO or PRMT5i treated samples as well as splice events that were differentially expressed by 4 days of PRMT5i treatment compared to DMSO (Δ AS: 4d PRMT5i vs DMSO) (Figure 4.7A). We saw very similar results between MKL-1 and WaGa cells with CASs being the most commonly detected splice type, followed by DIs, then A3PSS, A5PSS, and MXEs (Figure 4.7B). When we examined the proportion of AS events differentially expressed with PRMT5i, we saw in contrast that DIs were by far the splice type with the greatest number of significant changes in expression with PRMT5 inhibition. This finding shows that although DIs are not the most common splice type detected in MCC, DIs are the most affected by PRMT5 inhibition. When we plotted the distribution of the fold change for each alternative splice event, we saw that the vast majority of differentially expressed DIs were upregulated with PRMT5i treatment (Figure 4.7C). In contrast, the other differentially expressed splice events predominantly decreased with PRMT5i treatment in both WaGa and MKL-1 cells. These findings suggest that PRMT5 is required for removal of DIs in MCC cells.

Although the majority of DIs increased in both MKL-1 and WaGa, we wanted to determine whether the differentially expressed DIs with PRMT5i treatment were conserved between cell lines. We found that 2,721 out of the 2,900 detained introns differentially upregulated in MKL-1 cells (93.8%) were also upregulated in WaGa cells, suggesting that the detained introns regulated by PRMT5 are highly conserved between MCC lines (Figure 4.7D). We performed a gene ontology analysis using DAVID to characterize the genes that had a significant increase in detained introns with PRMT5i in both cell lines and found an enrichment for genes involved in protein transport,

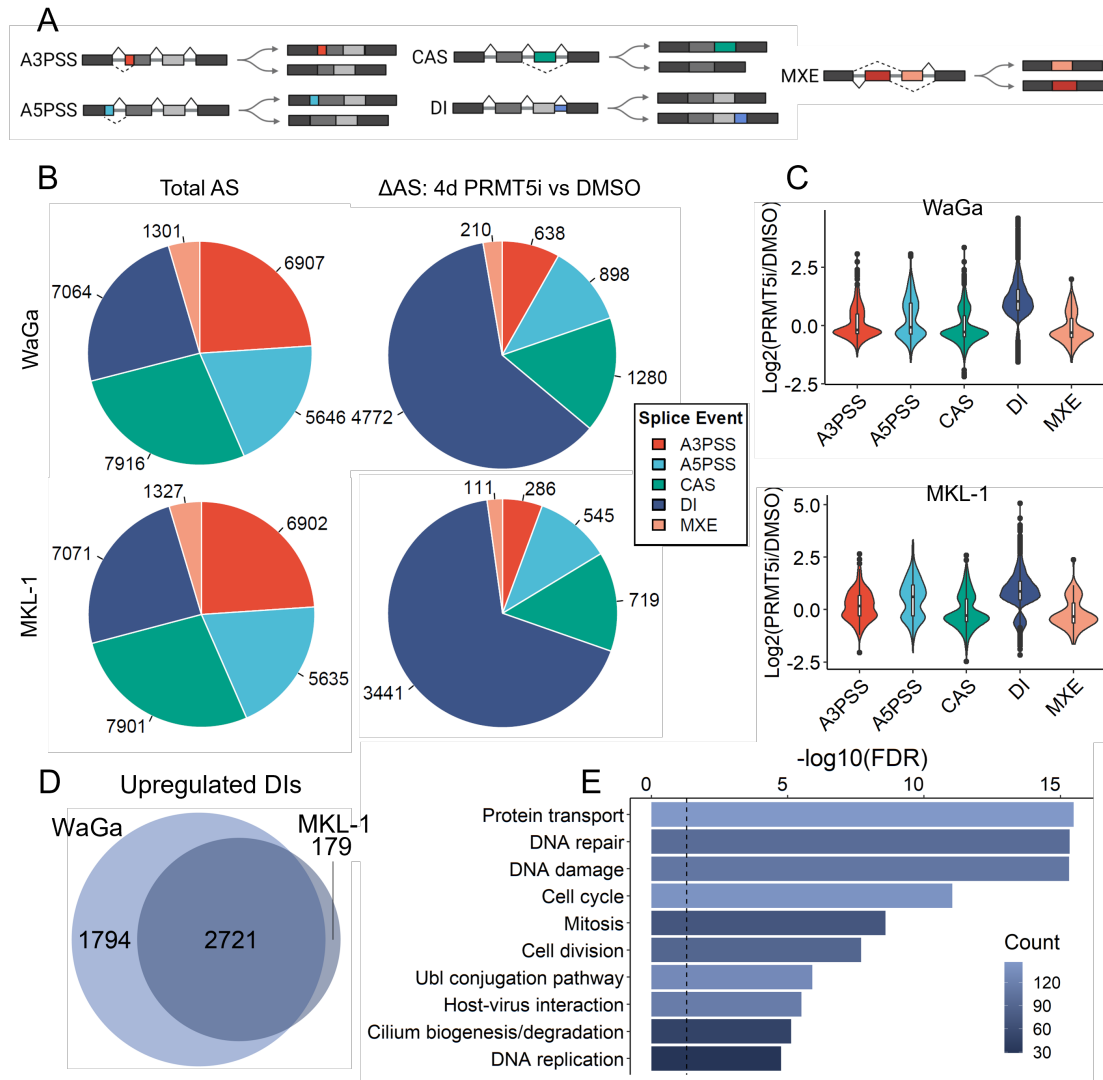


Figure 4.7: PRMT5 inhibition leads to an increase in detained introns of genes involved in cell cycle and DNA damage repair. **A.** Type of alternative splice events (AS) detected with DEXseq analysis of MCC RNA-seq data. A3PSS = alternative 3' splice site; A5PSS = alternative 5' splice site; CAS = cassette exon; DI = detained intron; MXE = mutually exclusive exon. Created with BioRender.com. **B.** Proportion of alternative splice events in WaGa and MKL-1 cells. Total AS = alternative splice events identified in either DMSO or PRMT5i treated samples. ΔAS: 4d PRMT5i vs DMSO = significantly ($p_{adj} < 0.05$) differentially expressed AS with 4 days PRMT5i treatment. **C.** Violin plot of log₂ fold change of differential AS in WaGa or MKL-1 treated with PRMT5i for 4 days. **D.** Venn diagram of differentially upregulated DIs ($p_{adj} < 0.05$) detected in WaGa and MKL-1 cells. **E.** DAVID gene ontology analysis of genes containing shared upregulated detained introns shared from D.

DNA repair/DNA damage, and cell cycle/mitosis (Figure 4.7E). Of the 108 DNA repair genes with significant increases in DIs, approximately one fifth (21) were part of the double strand break repair. These included key regulatory proteins of the homologous recombination repair pathway such as ATM, BRCA1, and BRCA2 as well as critical modulators of the non-homologous end joining pathway like DNA-PKcs (*PRKDC*), 53BP1 (*TP53BP1*), and Ku80 (*XRCC5*). We also saw there were a number of genes in the mitotic G2 DNA damage checkpoint GO term including CHK1 (*CHEK1*), TOPBP1, and claspin (*CLSPN*).

4.4.3 PRMT5 LOSS INCREASES PROTEIN EXPRESSION OF MRNA SPLICING FACTORS.

Based on the current understanding in the field that transcripts containing DIs are not translated¹¹⁴, we predicted from our data that PRMT5 loss would decrease expression of hundreds of genes, including many key regulators of multiple arms of the DNA damage response. To test this assumption and to gain a better understanding of the effect of PRMT5 loss on the DNA damage response, we performed quantitative mass spectrometry on MKL-1 and WaGa samples treated with 10 nM PRMT5i for 4, 8, and 12 days. We identified a total of 8,090 and 7,355 proteins in MKL-1 and WaGa cells respectively across treatment conditions, of which 6,774 (92% of WaGa) were shared between the two cell lines. We calculated the Z-scores for each protein within each cell line across treatment conditions for visualization (Figure 4.8A). Qualitatively, we saw that protein expression changes progressively intensified with treatment duration with the strongest effects seen after 12 days of treatment. There was not a total decrease or increase in protein expression; the effect of PRMT5i treatment on protein expression overall was split. We used linear modeling of each protein to calculate the significance of the protein expression change with PRMT5i treatment over time. To

assess the degree of similarity between cell lines, we compared the adjusted p values for each protein between MKL-1 and WaGa and found that the direction and significance of protein changes correlated relatively well between cell lines (Pearson correlation coefficient $R = 0.7$) (Figure 4.8B).

We examined what proportion of proteins were differentially expressed in MKL-1 and WaGa cells and found that more than 85% were significantly increased or decreased in at least one cell line with about half (3,346) shared in both cell lines (Figure 4.8C). Only 789 (approx. 12%) were unchanged in both MKL-1 and WaGa cells, and even fewer (approx. 5%) had contrasting expression changes with the protein increasing in one cell line but decreasing in the other. Together these findings indicate that treatment with PRMT5i over 12 days alters global protein expression.

To identify protein expression changes that were due to direct effects of PRMT5 loss, we focused in on the proteins that were differentially expressed after 4 days of treatment compared to DMSO. We identified 464 significantly upregulated proteins and 549 significantly downregulated proteins shared between MKL-1 and WaGa cells (Figure 4.9A). We performed GO term enrichment analysis with DAVID using the total proteins detected in MKL-1 and WaGa as background and found that factors involved in mRNA splicing and processing were significantly enriched among the proteins upregulated after 4 days of PRMT5i treatment, while proteins involved in cell division and mitosis were significantly downregulated (Figure 4.9B). We took a closer look at the significantly upregulated proteins involved in mRNA splicing and found that a number of these proteins were also identified in the CRISPR screen as negatively selected (Figure 4.9C). Based on these data, we propose a model where PRMT5 inhibitor treatment leads to an upregulation of splicing factors in response to splicing dysregulation due to loss of PRMT5 activity.

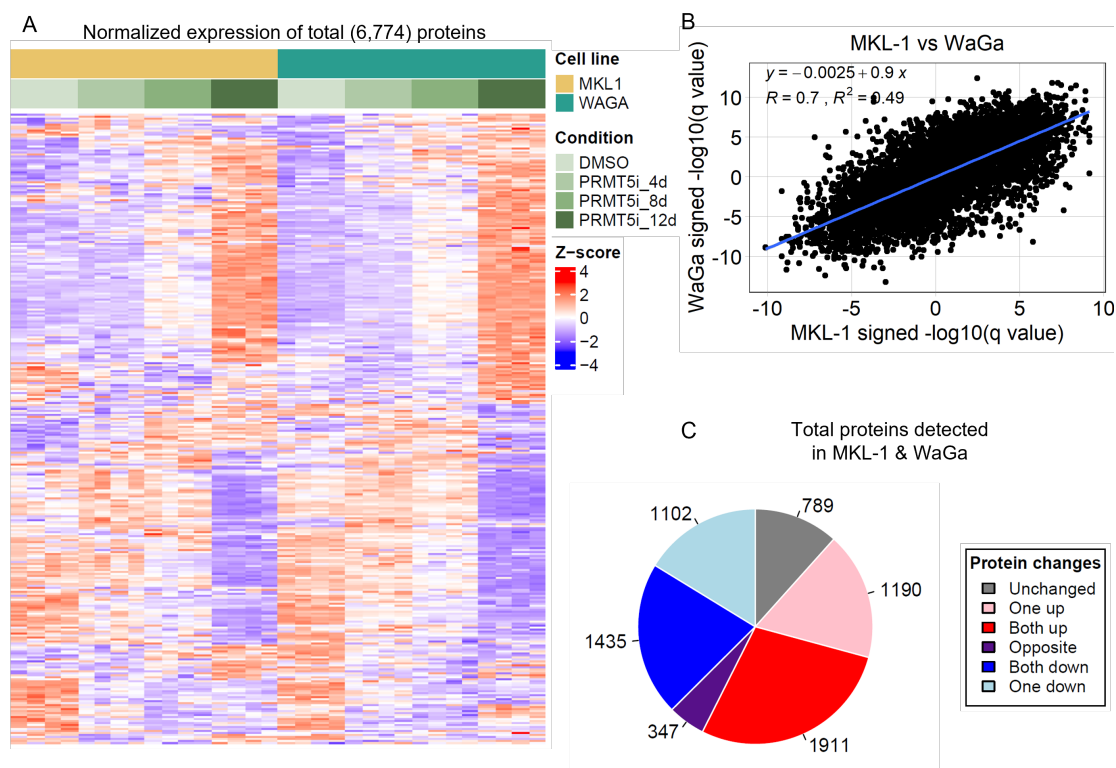


Figure 4.8: PRMT5 loss alters global protein expression. **A.** Heatmap of Z-scored total protein expression over a 12 day PRMT5i treatment timecourse in MKL-1 and WaGa cells in quadruplicate. **B.** Correlation between q values of proteins from MKL-1 or WaGa cells calculated using linear modeling comparing PRMT5i treatment over 12 days to DMSO. Q value = p value adjusted for false discovery with Benjamini-Hochberg procedure. Pearson correlation coefficients are shown. **C.** Pie chart of total proteins detected in both MKL-1 and WaGa cells treated with PRMT5i colored by significance and direction of drug effect. Unchanged = no significant change in either cell line; One up = significantly increased expression in only one cell line; Both up = significantly increased in both cell lines; Opposite = significantly increased in one cell line and significantly decreased in the other; Both down = significantly decreased in both cell lines; One down = significantly decreased in only one cell line.

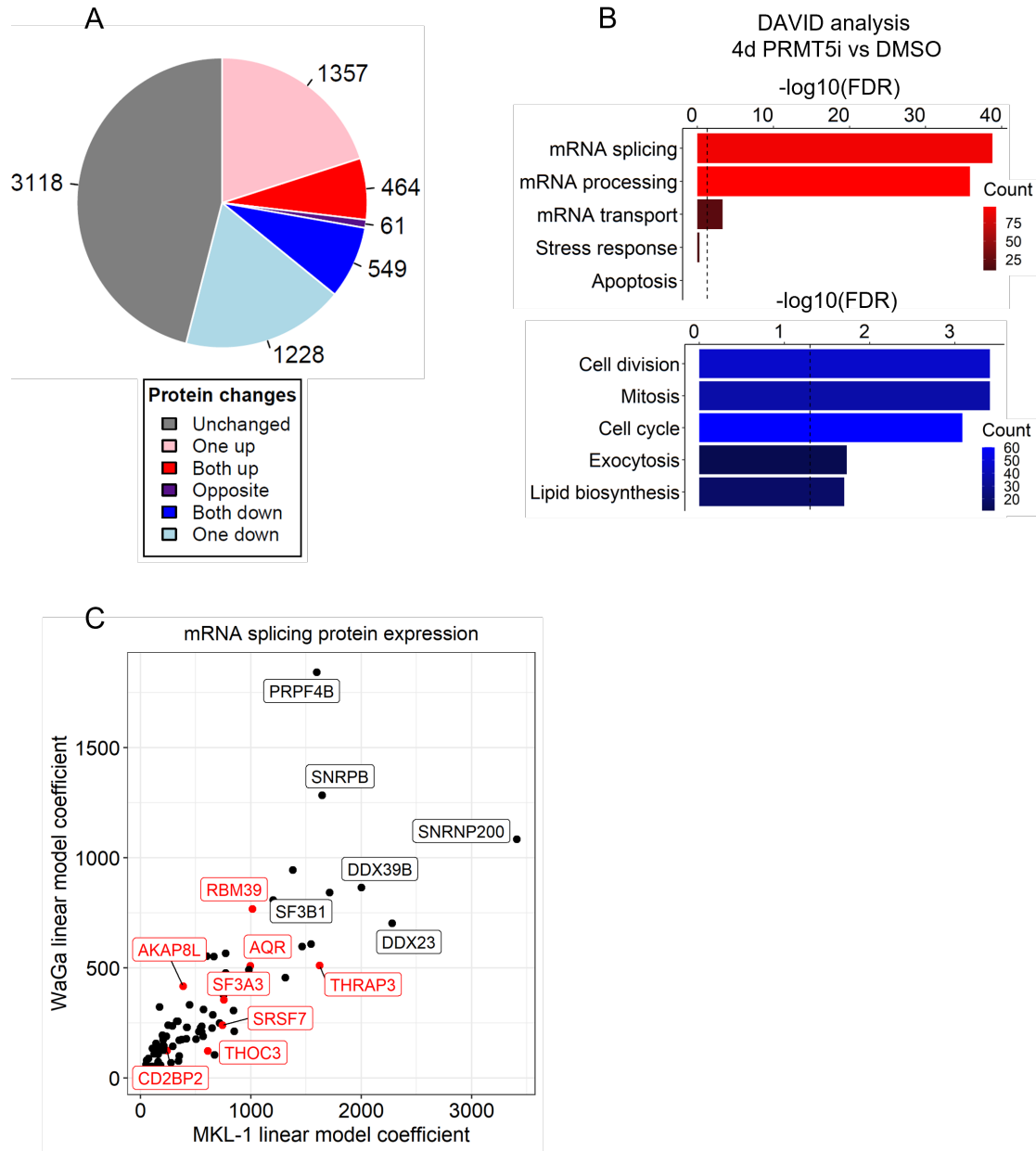


Figure 4.9: PRMT5 loss leads to upregulation of splicing factors. **A.** Pie chart of total proteins detected in both MKL-1 and WaGa cells treated with PRMT5i for 4 days colored by significance and direction of drug effect. **B.** Gene ontology enrichment analysis of significantly upregulated (red) and downregulated (blue) proteins shared between MKL-1 and WaGa after 4 days PRMT5i treatment using DAVID¹⁷⁵. **C.** Expression of proteins from mRNA splicing GO term in B. in MKL-1 vs WaGa. Negatively selected CRISPR hits are highlighted in red.

As mentioned above, an increase in DIs in a gene is expected to reduce levels of transcripts available for translation and therefore decrease protein expression. We hypothesized therefore that DI inclusion would correlate with protein expression down-regulation. To test this hypothesis, we compared changes in RNA to changes in protein with and without correcting for DIs in WaGa cells (Figure 4.10). Using EBseq, we approximated the level of "coding" transcripts by subtracting out the transcripts predicted to contain DIs. We asked whether correcting for increases in DIs would improve the correlation between changes in RNA and protein after 4 days PRMT5i treatment. We found that overall, the correlation between changes at the RNA level and changes at the protein level was quite low with a Pearson correlation coefficient $R = 0.17$ (Figure 4.10A). This is not entirely unexpected, as previous studies have found similarly low correlation coefficients between mRNA and protein expression data¹⁹⁴ and there are many known additional factors such as protein stability that contribute to protein expression. Interestingly, correcting for DIs did not improve correlation between the changes in RNA vs protein when including total proteins. When we focused in on DNA damage genes we identified as having DIs, we again found that the correlation between protein and RNA was very low (Pearson correlation coefficient $R = 0.029$) (Figure 4.10B). We saw that correcting for DIs improved the correlation, however it remained quite low ($R = 0.29$). We repeated this analysis for WaGa cells and saw the same trend, although the correlation of the DNA damage RNA expression vs protein expression was lower, with $R = -0.13$ before correcting for DIs and $R = 0.078$ after (data not shown). Overall, we conclude that higher levels of DIs may play a role in protein expression regulation but is not sufficient to predict protein expression changes in the context of PRMT5 inhibition.

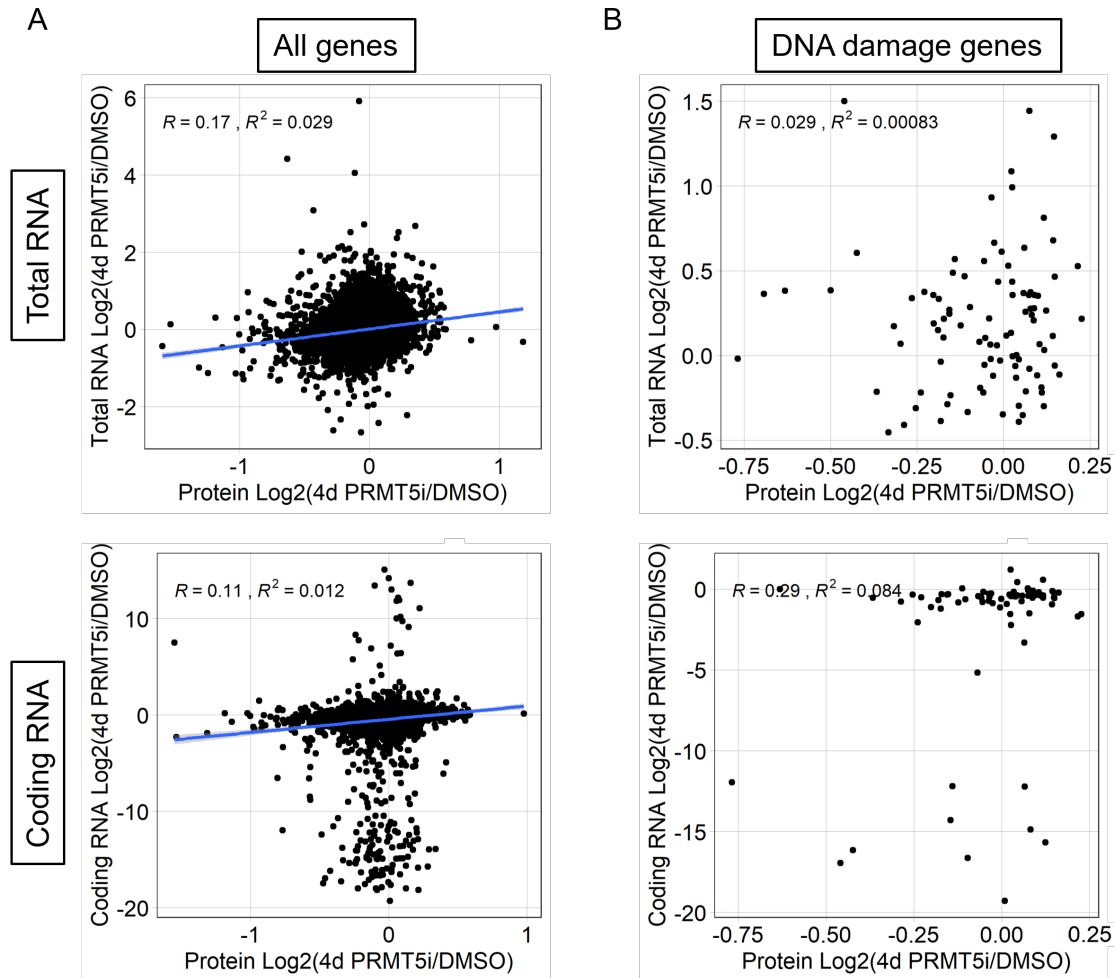


Figure 4.10: Correcting transcript levels for DIs improves correlation with protein expression for DNA damage genes. Log2 fold change of 4 day PRMT5i vs DMSO of RNA expression vs protein expression for all genes (A.) or DNA damage genes containing DIs (B.). Total RNA = mRNA expression changes calculated using DESeq; Coding RNA = mRNA expression changes corrected for DI levels using EBSeq.

4.5 DISCUSSION

In this chapter, we found that of all of the previously characterized PRMT5 targets and functions, the role of PRMT5 in mRNA splicing is the most critical to MCC growth. We performed a CRISPR screen in MKL-1 cells and found that genes involved in mRNA processing were enriched among the negatively selected hits, including splicing factors SF3A3, SRSF7, as well as previously identified PRMT5 methylation targets including SUPT5H and PAIP1. Interestingly, CLNS1A, the PRMT5 adaptor protein that promotes PRMT5 methylation of splicing factors, had a negative beta score difference while the other two known PRMT5 adaptors RIOK1 and COPR5 both had a positive beta score difference. It has been shown that these three adaptors all bind to the same region of PRMT5 and compete with one another for binding⁸³. These findings support a model where loss of COPR5 or RIOK1 expression allows for increased binding between CLNS1A and PRMT5 which could increase targeting of PRMT5 to splicing factors and provide resistance to PRMT5 inhibition. From these data, we concluded that regulation of mRNA splicing is a critical feature of PRMT5 activity in MCC.

We therefore characterized the effect of PRMT5i on the mRNA splicing by RNA-seq, using deep sequencing in order to perform an analysis of the alternative splice events altered by PRMT5 loss. We first examined the effect of PRMT5 inhibition on total RNA expression. Although we collected samples at 4 and 8 days with PRMT5i, we saw that most RNA expression changes occurred within 4 days of treatment. On a total transcript level, we found that there were differences in how PRMT5i affected MKL-1 and WaGa cells, namely that genes involved in cell cycle were specifically downregulated in WaGa. There was a higher degree of similarity of genes upregulated

with PRMT5i treatment between cell lines, however we weren't able to identify any specific gene signatures associated with these genes.

The effect of PRMT5 loss on mRNA splicing between cell lines was much more consistent, with PRMT5 inhibition leading to a significant increase in detained introns (DIs). Over 90% of the DIs upregulated in MKL-1 cells with PRMT5 inhibition were shared with WaGa cells, suggesting that splicing of these introns is highly conserved and dependent on PRMT5 activity. When we analyzed these genes further we saw that these included many genes involved in the DNA damage response. Previous work corroborates these results: Boutz et al.¹¹⁴ showed that DIs are a highly conserved class of spliced introns and found an enrichment of DIs in DNA damage genes. With regards to PRMT5 specifically, many studies have found that PRMT5 loss increases levels of detained introns and skipped exons^{74,99,75,169}, despite some discrepancies as to the level of one splice type vs the other. Our data suggests that PRMT5 primarily regulates splicing of DIs, although we do also see a decrease in cassette exon (CAS) usage. One explanation for how PRMT5 regulates both removal of DIs and inclusion of CASs is that PRMT5 is required for splicing of weak splice sites. This has been previously reported by a number of groups^{74,195} however Braun et al. found however that PRMT5 activity was not correlated with splice site strength⁹⁹. Future work is necessary to identify additional features or mechanisms dictating PRMT5 splice site regulation.

We also characterized the effect of PRMT5 inhibition on the proteome using quantitative mass spectrometry. We saw that over a 12 day treatment, PRMT5 inhibition altered expression of thousands of proteins, at least half of all detected proteins. Many of these changes are likely due to indirect effects of PRMT5 loss, and in Chapter 3 we saw that most of our cells were apoptotic after 12 days of treatment, so to iden-

tify more direct effects we focused on changes in protein expression after 4 days of PRMT5i treatment. We saw that among the differentially expressed proteins shared between MKL-1 and WaGa cells, proteins involved in mRNA splicing significantly increased while proteins involved in cell division significantly decreased after 4 days of treatment. We found that of these upregulated splicing factors, many were also identified as negatively selected in the MKL-1 CRISPR screen. One model to explain this finding is that the cell attempts to correct the splicing defects observed with PRMT5 loss through upregulation of splicing factors, although additional work is necessary to identify a mechanism for how the cell senses splicing defects.

By performing both RNA sequencing and total protein quantitative mass spectrometry under the same conditions, we were able to examine the relationship between RNA and protein expression changes in PRMT5i-treated MCC cells. We found that the correlation between changes in total RNA vs total protein was quite low, and this correlation was not improved even when correcting for DI-containing transcripts overall. When we focused more specifically on genes whose splicing was affected by PRMT5i treatment, namely genes involved in the DNA damage response, we saw that correcting for DIs did improve the correlation between RNA and protein level changes slightly, although the overall correlation was still quite low. Altogether, these findings show that an increase in DIs is not sufficient to cause a decrease in protein expression, as transcript level is just one factor of many influencing protein expression.

AUTHOR CONTRIBUTIONS FOR CHAPTER 5

The three-color flow cytometry experiments were performed by Camille H. Cushman with guidance from Hembly G. Rivas. The DSB reporter assay and all associated data in Figure 5.3 was performed by Mona M. Ahmed. The immunoblot experiments in Figure 5.4A-C were performed by Mona M. Ahmed. All other experiments were conceptualized and performed by Camille H. Cushman with input from James A. DeCaprio.

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5

Investigating the role of PRMT5 in regulating the double strand break and replication stress response.

5.1 ABSTRACT

PRMT5 has previously been shown to regulate genome stability through methylation and mRNA splicing of DNA damage proteins. Our data from Chapter 4 showed

in MCC cells specifically that PRMT5 regulated splicing of detained introns from DNA damage response genes. In this chapter, we showed that PRMT5 loss induces replication-associated double strand breaks (DSB) in sensitive MCC cell lines but not in resistant lines. We found that expression of both ATM and ATR, key regulatory kinases involved in the DSB and replication stress response, is diminished with PRMT5 inhibition. We investigated the effect of PRMT5 loss on the DSB response and found that PRMT5 loss promotes repair by NHEJ but not repair by HR. We showed that the ATR/CHK1 response to both irradiation (IR) and hydroxyurea (HU) treatment was diminished, leading to higher levels of replication stress as measured by RPA32 hyperphosphorylation. We determined that DNA-PKcs was able to regulate phosphorylation of both RPA32 and CHK2 when cells were treated with HU combined with PRMT5i. Ultimately we conclude that the replication-associated DNA damage observed with PRMT5 inhibition may be exacerbated by an inability of the ATR/CHK1 pathway to respond to replication stress.

5.2 INTRODUCTION

Understanding how PRMT5 inhibition causes cell death has been a surprisingly complicated question. Although previous studies have found that activation of the p53 response with PRMT5 inhibition induces apoptosis, our work has shown that p53 loss is not sufficient to rescue PRMT5 toxicity in MCC. This finding is consistent with reports from other groups as well⁷⁶. We do see a p53 response with PRMT5 inhibition, so it is possible that PRMT5i triggers a p53 response partially due to an accumulation of DNA damage. Multiple groups have shown that PRMT5 loss leads to increased DNA damage^{76,196}, and others have shown that high levels of unresolved DNA dam-

age can lead to p53-independent apoptosis (reviewed in ¹⁹⁷).

There are a few models in the field to explain how PRMT5 loss causes DNA damage. One theory is that PRMT5 is required for regulating expression or activity of proteins involved in the DNA damage response. Normal cells experience intrinsic DNA damage from sources such as replication errors, DNA lesions like R-loops, and from exposure to endogenous chemicals like reactive oxygen species (reviewed in ¹⁹⁸). An inability of the cell to properly respond to intrinsic DNA damage can lead to an accumulation of DNA breaks. PRMT5 has been shown to play a role in the DNA damage response (DDR), particularly the double strand break (DSB) response, through both changes in alternative splicing and direct methylation of proteins involved in DSB repair (reviewed in ⁸⁰). An alternative theory is that PRMT5 is required for R-loop resolution which has reported by a number of groups ^{199,200} and as mentioned above can be a source of intrinsic DNA damage. It's possible that both models are true and contribute to genome instability with PRMT5 inhibition. Here, we will briefly summarize current knowledge of the major pathways involved in responding to DSBs before discussing in greater detail what is known about the role of PRMT5 in DDR regulation.

5.2.1 DOUBLE STRAND BREAK (DSB) REPAIR

Although there are numerous types of DNA damage, the most cytotoxic DNA lesions are thought to be DSBs (reviewed in ^{201,202}). DSBs can be repaired by a few different mechanisms that are differentiated based on whether they use a homologous DNA sequence as a template. Non-homologous end joining (NHEJ) involves direct ligation of the broken ends back together without the need for an additional template sequence. In contrast, homologous recombination (HR) requires a template sequence (typically the sister chromatid) and involves 5'-3' end resection. The single-stranded 3' end is

then used for template search and recombination. An early response to the DSB includes recruitment and activation of ataxia telangiectasia mutated protein (ATM), a kinase in the phosphoinositide 3-kinase (PI3K)-related kinases (PIKK) family, that initiates a signaling cascade to facilitate chromatin remodeling and DNA end recognition. This signaling cascade typically includes phosphorylation of H2AX on serine 139 to form “ γ -H2AX chromatin domains” which function to recruit additional DDR proteins, and the presence of γ -H2AX is often used as a marker for DSB repair.

The decision between repair pathways depends in part upon the type of break and the cell-cycle phase. If the break contains a long single-stranded DNA (ssDNA) tail, then HR is favored. If not, Ku70 (*XRCC6* gene) and Ku80 (*XRCC5* gene) will bind to the ends of the break and the cell-cycle phase will be considered. NHEJ is favored during G1, and the next steps in the NHEJ pathway include 53BP1 recruitment and activation, DNA-dependent protein kinase catalytic subunit (DNA-PKcs: another PIKK) recruitment to Ku70-Ku80, and ligation of the DNA ends by DNA ligase IV (LIG4). HR is largely restricted to S and G2 due to the requirement for a template sequence for repair. The next steps in HR include BRCA1 binding, DNA end resection, Ku70-Ku80 displacement, RPA binding to ssDNA, RPA displacement by RAD51, and recombination mediated by BRCA2 and PALB2 (partner and localizer of BRCA2). More specifically, BRCA1 recruits BRCA2 and PALB2 to the DSB to promote replacement of RPA with RAD51 which facilitates DNA strand invasion and ultimately recombination. In addition to these major repair pathways, there exist error-prone repair pathways including alternative end joining (aEJ) and single strand annealing (SSA). It is thought that all DNA repair pathways may compete for access to any and all DNA ends. Regulation of the binding and recruitment of DDR proteins to the DSB is essential to ensure that the break is repaired successfully prior to cell division.

The genome is thought to be particularly susceptible to damage during DNA replication, as the entire genome must be unravelled and faithfully duplicated by DNA helicases and polymerases. Direct barriers to DNA replication such as secondary DNA structures, R-loops, and nucleotide imbalances can lead to altered replication fork progression, an increase in replication errors, and DSBs. Together, these phenomenon are referred to as replication stress (reviewed in²⁰³). Replication stress can lead to genomic instability and if the cell is unable to respond to high levels of replication stress this can lead to replication catastrophe and cell death.

Ataxia telangiectasia and Rad3 related protein (ATR, another member of the PIKK family) is thought to be the major kinase responsible for coordinating the replication stress response to regulate DNA damage repair and cell cycle progression in what is considered the S-phase checkpoint (reviewed in²⁰⁴). During DNA replication, if the polymerase encounters a DNA lesion it will stall, leading to uncoupling of the polymerase with the helicase. As the helicase continues to separate DNA strands, long tracts of ssDNA are produced and rapidly coated with RPA. ATR-interacting protein (ATRIP) recognizes RPA-bound ssDNA tracts and recruits ATR to the site of the stalled polymerase. ATR is then activated by association with TOPBP1 and phosphorylates RPA. Another protein called claspin (*CLSPN*) promotes ATR-mediated phosphorylation and activation of CHK1 (*CHEK1*). Activated CHK1 then promotes degradation or sequestration of CDC25A leading to inhibition of cyclin-dependent kinases (CDKs) and a slowing or arrest of the cell cycle. This allows for recruitment of DSB repair machinery, typically proteins involved in repair by HR, and repair of the break prior to cell division. If the cell divides with broken DNA strands, this can lead to improper chromosome segregation and mitotic catastrophe, a mode of cell death or irreversible cell cycle arrest caused by premature cell division (reviewed in^{205,206}).

5.2.2 ARGININE METHYLATION BY PRMT5 IN REGULATING DSB REPAIR

The response to DSBs is a highly complex set of signaling pathways that relies on post-translational modifications to coordinate protein recruitment and activities. The role of kinases and serine/threonine phosphorylation, particularly by the PIKK family of kinases, has been appreciated for many years and is summarized briefly above. Arginine methylation is more recently being appreciated as playing crucial roles in regulating the DDR as well. The role of PRMT5 in the DDR in particular is an area of active research. Multiple groups have shown that PRMT5 inhibition leads to defects in repair of DSBs by HR, leading to increased sensitivity to DNA damaging agents like gemcitabine²⁰⁷, IR^{164,116}, hydroxyurea¹⁶⁴ and PARP inhibitors⁷⁶.

A number of models have been proposed to explain how arginine methylation by PRMT5 impacts DSB repair decision. One model for the role of PRMT5 in promoting HR is through safeguarding the splicing of DDR proteins. Multiple groups have attributed PRMT5 regulation of the DDR to splicing of DDR genes more broadly^{99,169} whereas others have focused on splicing of specific DDR proteins like TIP60⁷⁶. An alternative model is that PRMT5 directly methylates proteins that influence the DDR, like RUVBL1¹¹⁶ or Rad9¹⁶⁴.

Here, we investigated the role of PRMT5 in regulating the response to DSBs in MCC. We first asked whether PRMT5 loss induced DNA damage and found that treatment with PRMT5 inhibitor induced DNA damage in sensitive MCC lines but not in a resistant line. By taking a cell cycle phase-specific approach, we found that marks of DSBs accumulated first in S phase indicating that PRMT5 inhibition caused replication-associated DSBs. To determine whether PRMT5 loss also influences the response to DNA damage, we investigated both the DSB response and replication stress re-

sponse using DNA damaging agents. In response to double strand breaks, we found that PRMT5i promoted repair by NHEJ and regulated the splicing and expression of a number of key DNA repair genes including ATM. In response to replication stress, we found that PRMT5 loss induced an ATR/CHK1 activation defect and DNA-PKcs activation. Altogether, we provide evidence that DNA damage induced by PRMT5 loss contributes to sensitivity in MCC, and show that both the DSB and replication stress response are dependent on PRMT5 activity.

5.3 METHODS

5.3.1 CELL CULTURE

MCC cell lines were gifts from Masahiro Shuda (University of Pittsburgh), Jürgen Becker (Translational Skin Cancer Research, German Cancer Consortium (DKTK), Essen, Germany), and Roland Houben (University of Wuerzburg, Germany). Cells were cultured in RPMI (Life Technologies, 11875119) with 10% FBS and 1% penicillin-streptomycin (Life Technologies 15140163). Cell lines were confirmed to be free of mycoplasma contamination using the e-Myco Mycoplasma PCR Detection Kit (Bulldog Bio 25233).

5.3.2 IMMUNOBLOTTING

Cell pellets were lysed in EBC buffer and protein content was quantified by Bradford assay. Lysates were separated on either a 4-20% or 4-12% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked in 5% milk, and incubated with primary antibody at 4°C overnight. A list of the primary antibodies used can be found in Table 5.1. Blots were imaged with either SuperSignal West Pico (ThermoFisher, PI34080)

or Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500).

Table 5.1: Antibodies used for immunoblot

Target	Catalog Number
53BP1	Abcam ab36823
53BP1 pS25	Abcam ab70323
ATM	CST 2873S
ATM pS1981	CST 4526S
ATR	CST 13934S
ATR pT1989	CST 30632S
CHK1	CST 2360S
CHK1 pS317	CST 8191S
CHK1 pS345	CST 2348S
CHK2	CST 3440S
CHK2 pT68	CST 2197S
DNA-PKcs	CST 38168S
DNA-PKcs pS2056	CST 68716S
H2AX pS139	Sigma Aldrich 05-636-I
KAP1	Bethyl A300-274A
KAP1 pS824	Bethyl A300-767A
MDM4	Abcam ab243859
PARP	CST 9542S
RPA32	Bethyl A300-244A
RPA32 pS4/8	Bethyl A300-245A
RPA32 pS33	Bethyl A300-246A
SDMA	CST 13222S
VINC	Sigma Aldrich V9131

5.3.3 THREE-COLOR FLOW CYTOMETRY

Flow cytometry experiments were performed as previously described¹⁵¹. Briefly, cells were treated with 10 uM EdU (Click Chemistry Tools, 1149-100) for 1 hour prior to collection. Cells were fixed in 4% formaldehyde in PBS for 15 minutes at room temperature before being permeabilized in 70% ethanol at -20°C overnight. Incorporated EdU was labeled using a CLICK reaction (2 mM CuSO₄, 100 M THPTA,

100 mM sodium ascorbate, and 2 M Alexa 647 Azide in PBS) by rotating for 30 minutes at room temperature protected from light. Samples were incubated with primary antibody using a dilution based on the antibody manufacturer's recommendations overnight at 4°C protected from light. Samples were washed in 1% BSA and incubated with PE-conjugated secondary antibodies for 1 hour at room temperature protected from light. Antibodies are listed in Table 5.2. DNA was stained with a 1 ug/mL DAPI, 100 ng/mL RNase A staining solution for 1 hour at room temperature protected from light prior to analysis by flow cytometry (BD LSRFortessa) at the Dana-Farber flow cytometry hematologic neoplasia and Jimmy fund core.

Table 5.2: Antibodies used for flow cytometry

Target	Catalog Number
cleaved caspase-3	CST 9661S
H2AX pS139	Sigma Aldrich 05-636-I
PE conjugated anti-mouse IgG secondary	CST 8887S
PE conjugated anti-rabbit IgG secondary	CST 79408S

5.3.4 DSB REPORTER ASSAY

CELL CULTURE

U2OS DR-GFP and EJ5-GFP cell lines were gifts from Jeremy Stark (City of Hope). Cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) with 4.5 g/L glucose, L-glutamine without sodium pyruvate (Corning 10-017-CV) supplemented with 10% FBS (MilliporeSigma) and 1% penicillin-streptomycin (Thermo Fisher). Cell lines were confirmed to be free of mycoplasma contamination using the e-Myco Mycoplasma PCR Detection Kit (Bulldog Bio, Portsmouth, NH).

GFP-BASED REPORTER ASSAY

To perform the assay, cells were plated in 10 cm TC-treated plates at 50% confluency. The next day, 2 L of I-SceI adenovirus was added to the cell culture media. The following day, cells were washed with PBS and fresh media without I-SceI was added to cells. Cells were incubated at 37°C in a cell culture incubator for two days. Cells were harvested and fixed in 70% ethanol for 2-24 hours. GFP expression was measured by flow cytometry (BD LSRFortessa) at the Dana-Farber flow cytometry hematologic neoplasia and Jimmy fund core.

The appropriate amount of I-SceI adenovirus to add per plate was determined by infecting cells with increasing amounts of adenovirus and measuring GFP expression. After generating a stock of I-SceI adenovirus, I determined that 2 L was sufficient to induce the maximum amount of GFP expression while minimizing the amount of cell death. If a new stock of I-SceI adenovirus is made, the appropriate amount of virus to add should be re-determined. The protocol for the amplification of the I-SceI adenovirus has previously been previously described²⁰⁸.

5.4 RESULTS

5.4.1 INHIBITION OF PRMT5 ACTIVATES A DNA DAMAGE RESPONSE IN SENSITIVE MCC CELL LINES.

Understanding the determinants of sensitivity to PRMT5 inhibitors is a challenging question and likely depends on the cellular context. In Chapter 3, we found that in MCC, p53 status is not sufficient to predict sensitivity to PRMT5 loss and neither are PRMT5 levels nor SDMA response to PRMT5i. In Chapter 4, we found that PRMT5 regulation of mRNA splicing is a critical feature of PRMT5i sensitivity and

showed that PRMT5 regulates splicing of genes involved in the DDR, many of which were involved in the response to DSBs. Previous work has shown that loss of proper mRNA splicing can increase genomic instability^{209,210} and more specifically Hamard et al. found that PRMT5 inhibition induced DNA damage in a sensitive AML cell line but not in a resistant line⁷⁶. We therefore asked whether the same was true in MCC: specifically, whether PRMT5i treatment induced DNA damage in PRMT5i sensitive cells versus a resistant line. We first treated WaGa cells (a sensitive MCCC line) and MS-1 cells (a resistant MCCC line) with PRMT5i for 4 days and looked for evidence of DNA damage and response by immunoblot (Figure 5.1A). We saw that PRMT5i treatment induced a strong γ -H2AX signal in WaGa cells but not MS-1 cells. We also saw evidence of KAP1 phosphorylation which is a marker of ATM or DNA-PKcs activation in WaGa, suggesting that PRMT5 inhibition triggers a DNA damage response in PRMT5i-sensitive cells.

We also blotted for ATM, DNA-PKcs, and ATR, both phosphorylated and total protein, to gain a better understanding of which DNA damage response pathways were activated or inhibited by PRMT5i treatment. Phosphorylation of these kinases is typically considered a mark of kinase activation. We saw no evidence of ATM phosphorylation (data not shown) but we did see loss of total ATM protein with PRMT5i treatment in both WaGa and MS-1 cells. From the data collected in Chapter 4, ATM was identified as a gene with increased levels of DIs and reduced protein expression with PRMT5i treatment, so this finding is in alignment with our previous work. When we blotted for DNA-PKcs phosphorylation, we saw a band of about 300 kDa in the PRMT5i-treated WaGa cells alone. DNA-PKcs is predicted to be about 465 kDa, much larger than what we observed, so this band is likely not specific to DNA-PKcs. Total DNA-PKcs appeared to be unaffected by PRMT5i in either WaGa or MS-1 cells.

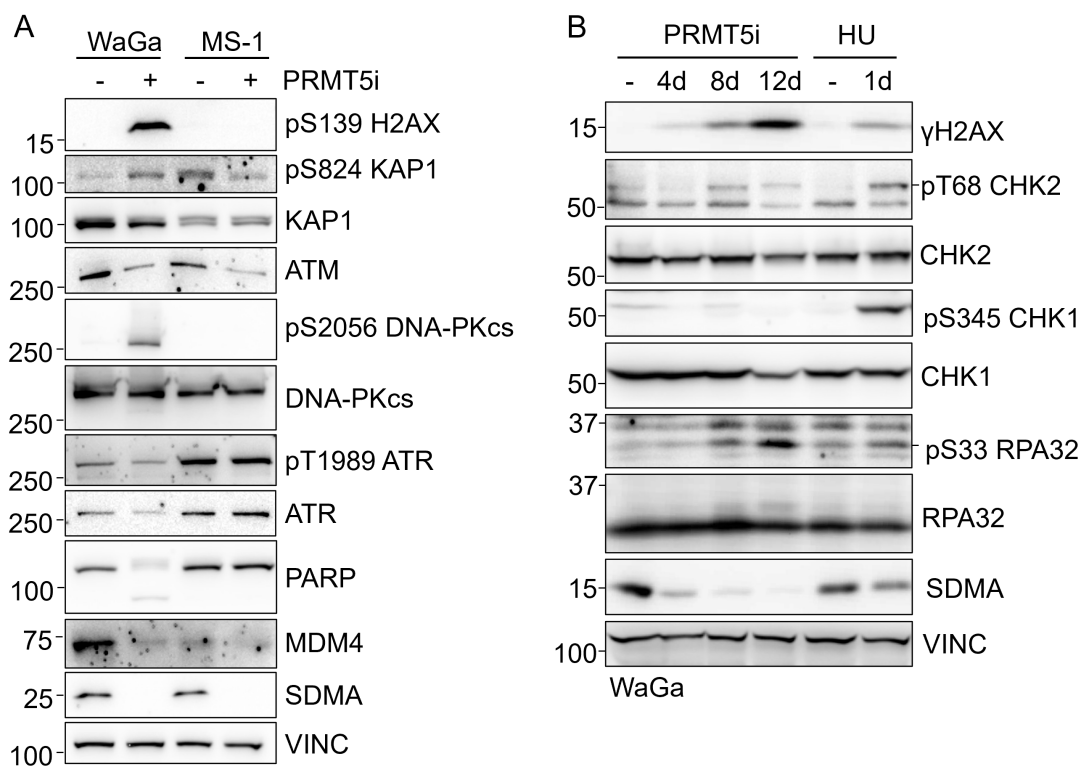


Figure 5.1: PRMT5i induces DNA damage in sensitive MCCP cells. **A.** Immunoblot of WaGa and MS-1 cells treated with 10 nM PRMT5i for 4 days. **B.** Immunoblot of WaGa cells treated with either 10 nM PRMT5i for 4, 8, or 12 days or 500 uM hydroxyurea for 1 day compared to a DMSO control.

Although DNA-PKcs was also identified in our DI analysis from Chapter 4, our proteomic analysis suggested that it was upregulated with PRMT5i treatment in WaGa cells, although this increase isn't detectable by immunoblot. When we blotted for total and phosphorylated ATR, we saw that PRMT5i caused a slight decrease in ATR expression in WaGa cells but had no effect on ATR expression or phosphorylation in MS-1 cells. We also blotted for PARP and saw evidence of PARP cleavage, a marker of caspase activation and apoptosis in PRMT5i-treated WaGa cells. MDM4 expression and SDMA levels were lost with PRMT5i treatment in alignment with our data from Chapter 3.

We next looked at the effect of PRMT5i treatment over time in WaGa cells and saw that γ -H2AX levels progressively increased with PRMT5i treatment length (Figure 5.1B). We blotted for CHK2 phosphorylation, a mark typically associated with ATM activity, and saw CHK2 phosphorylation levels moderately increase after 8 days of PRMT5i treatment. It is possible that the remaining ATM protein is activated after 8 days of PRMT5i treatment; another explanation is that ATR or DNA-PKcs phosphorylate CHK2 in response to PRMT5i, as these kinases have also been reported to phosphorylate CHK2 at T68 under certain DNA damage conditions²¹¹. We also blotted for phosphorylated CHK1 and phosphorylated RPA32, both targets of ATR. Interestingly, we saw no evidence of CHK1 phosphorylation but we did see increasing levels of phosphorylated RPA32 over time with PRMT5i treatment. Phosphorylation of RPA32 at S33 is thought to be primarily an ATR-specific event which leads to sequential phosphorylation of S4/S8 by DNA-PKcs²¹² generating a hyperphosphorylated, slower-migrating RPA32 band by immunoblot (reviewed in²¹³). The hyperphosphorylation of RPA32 is thought occur in response to replication-associated DSBs and play a role in stimulating DNA synthesis during repair of the break²¹⁴. These re-

sults suggest that PRMT5i induces DNA damage, but whether this damage is associated with replication is unclear due to the presence of hyperphosphorylated RPA32 without activation of CHK1 or ATR.

To determine whether PRMT5 loss causes replication-associated DSBs, we treated WaGa, MKL-1, and MS-1 cells with PRMT5i over time and analyzed cell cycle distribution and γ -H2AX levels by flow cytometry (Figure 5.2A-B). We saw that γ -H2AX levels increased over time with PRMT5i treatment in S phase MKL-1 and WaGa cells more than in any other cell cycle phase. There was a slight increase in γ -H2AX levels in S phase MS-1 cells, however this increase only reached about 2-fold whereas γ -H2AX levels reached 15- and 30- fold increases in MKL-1 and WaGa replicating cell respectively.

One confounding factor is that γ -H2AX has also been detected in non-DSB contexts, particularly with caspase activation during apoptosis^{215,216}. We therefore stained for cleaved caspase-3 in parallel in order to determine whether we could detect γ -H2AX signal prior to apoptosis. We found that the γ -H2AX signal in G1 correlated almost exactly with cleaved caspase-3, suggesting that the G1-specific γ -H2AX signal is primarily associated with apoptosis. The γ -H2AX signal in S phase cells however began increasing almost immediately after PRMT5i treatment while caspase-3 cleavage remained low. γ -H2AX levels in G2/M cells seemed to increase at early time points as well but the signal was not as strong and was less consistent than in S-phase cells. Overall, we conclude that PRMT5 inhibition leads to an increase in replication-associated DSBs prior to apoptosis.

5.4.2 PRMT5 IS REQUIRED FOR ATM AND ATR RESPONSE TO DNA DAMAGE.

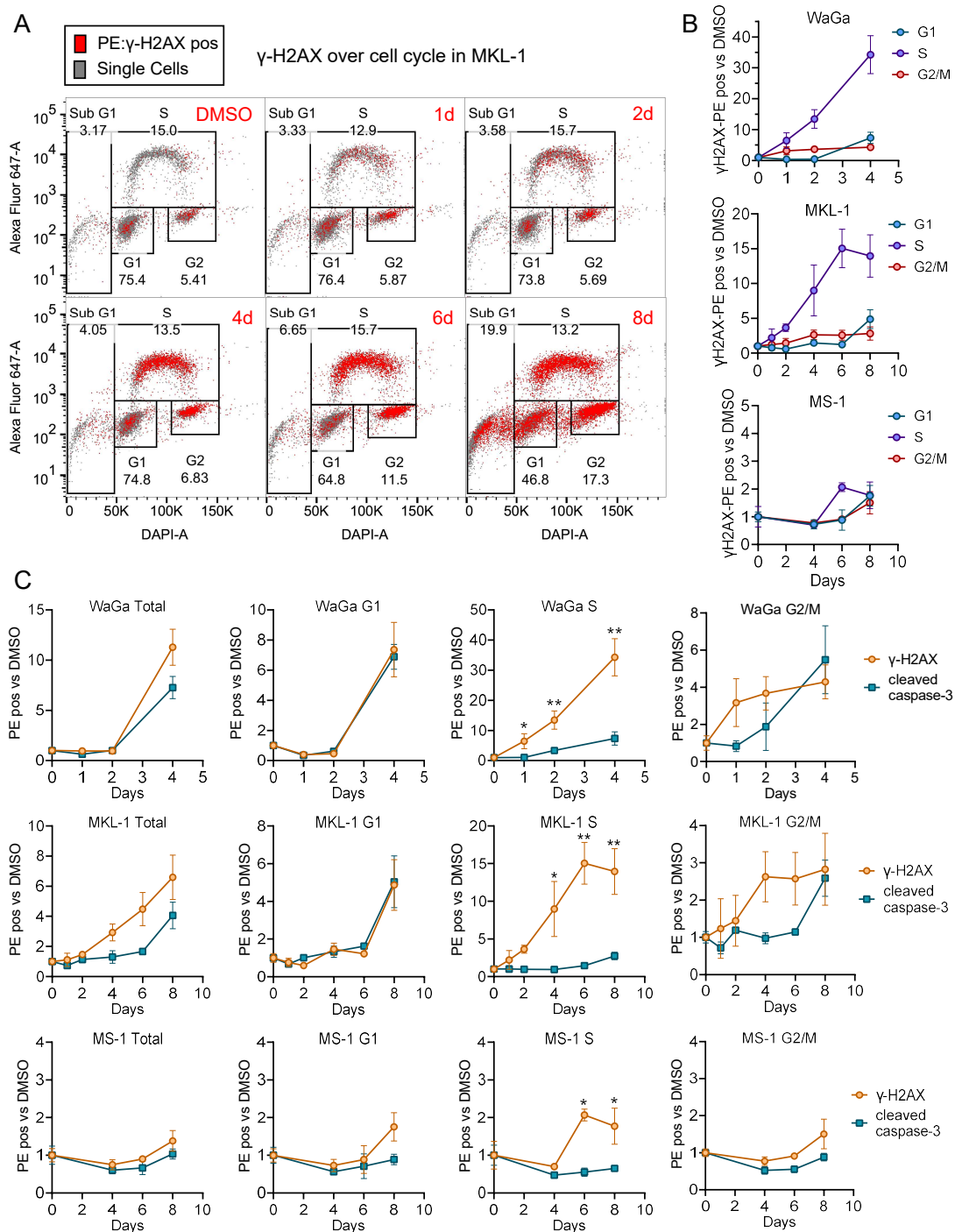
Replication-associated DSBs can be formed during normal cell division when a replication fork encounters a DNA lesion, leading to fork stalling and potentially fork collapse (reviewed in²¹⁷). Fork collapse often leads to the formation of single-end DSBs (seDSBs) which are preferentially repaired by HR. Repair of seDSBs by NHEJ mechanisms can lead to gene translocations when two seDSBs are inappropriately ligated together. Replicating polymerases encounter thousands of DNA lesions per cell even under normal, unstressed conditions, highlighting the importance of maintaining S-phase cell cycle checkpoints and regulation of DDR pathways during replication.

A number of groups have found that PRMT5 regulates the DSB response with loss of PRMT5 activity associated with a defect in homologous recombination (HR) repair^{76,116}. One technique to measure how a treatment condition affects the DSB repair pathway decision is by using fluorescence-based reporter assays. Gunn and Stark generated reporter U2OS cell lines containing GFP genes modified so that GFP is only expressed after a DSB has been introduced by the endonuclease I-SceI and repaired by either HR or NHEJ²¹⁸. The DR-GFP cell line expresses GFP after repair by HR whereas EJ5-GFP cells express GFP after repair by NHEJ. The level of GFP expression therefore correlates with how efficiently the GFP gene is repaired by either HR or NHEJ respectively.

Using this system, we tested the effect of PRMT5i treatment on the DSB repair pathway decision (Figure 5.3). We first confirmed that treatment with 10 nM of PRMT5i for 4 days effectively reduced SDMA levels in U2OS cells by immunoblot (Figure 5.3A). Cell cycle phase can affect the DSB repair pathway decision, so we next asked whether PRMT5i treatment altered the cell cycle distribution of U2OS cells and found

Figure 5.2 (following page): PRMT5i induces replication-associated γ -H2AX prior to caspase activation. Flow cytometry analysis of γ -H2AX and cleaved caspase 3 by cell cycle phase in MCCC cells treated with 10 nM PRMT5i over time. **A.** Representative flow plot of PE: γ -H2AX positive cells overlaid on cell cycle distribution of MKL-1 cells. **B.** Fold change of % PE: γ -H2AX positive cells within each cell cycle phase normalized to DMSO for WaGa, MKL-1, and MS-1 cells. N=2 for MKL-1 1d, 2d, and MS-1 6d; otherwise N=3. **C.** Fold change of % PE positive cells stained with either γ -H2AX (from B.) or cleaved caspase-3 within each cell cycle phase normalized to DMSO. N=2 for cleaved caspase 3 in MKL-1 and 6d MS-1; otherwise N=3.

Figure 5.2: (continued)



no significant differences after 4 days of treatment (Figure 5.3B). To determine whether PRMT5 loss impacted DSB repair decision, we pre-treated the reporter U2OS cell lines with PRMT5i for 3 days before inducing the DSB with I-SceI expression. We treated with PRMT5i for an additional 3 days before collecting for analysis by flow cytometry (Figure 5.3C-D). We found using the DR-GFP lines there was a slight decrease in GFP expression with PRMT5i treatment although this change was not statistically significant. We did see a significant increase in GFP expression in the EJ5-GFP reporter cells with PRMT5i treatment suggesting that PRMT5-deficient cells favor DSB repair by NHEJ compared to control cells.

To better characterize the effect of PRMT5i treatment on the DSB repair pathway in MCC, we used irradiation (IR) to induce DSBs and measured the effect of PRMT5i treatment on DSB repair pathway activation by immunoblot (Figure 5.4A). We saw little to no difference in DNA-PK, KAP1, or 53BP1 phosphorylation in PRMT5i treated MKL-1 cells compared to control. However we did see a reduction in ATR and CHK1 phosphorylation with PRMT5i treatment, suggesting there is a defect in the ATR/CHK1 response to DNA damage. We repeated this experiment using WaGa cells and again found a striking CHK1 phosphorylation defect, although the ATR activation defect was less pronounced (Figure 5.4B).

As mentioned earlier, ATR is the PIKK thought to be primarily responsible for coordinating the response to replication stress²⁰⁴. We asked more directly whether PRMT5i altered the replication stress response by treating MKL-1 and WaGa cells with PRMT5i and hydroxyurea (HU), a ribonucleotide reductase inhibitor that depletes nucleotide pools causing fork stalling during replication (Figure 5.4C-D). We found that PRMT5i reduced CHK1 phosphorylation levels in both MKL-1 and WaGa cells in response to HU treatment, suggesting that ATR activation was reduced with

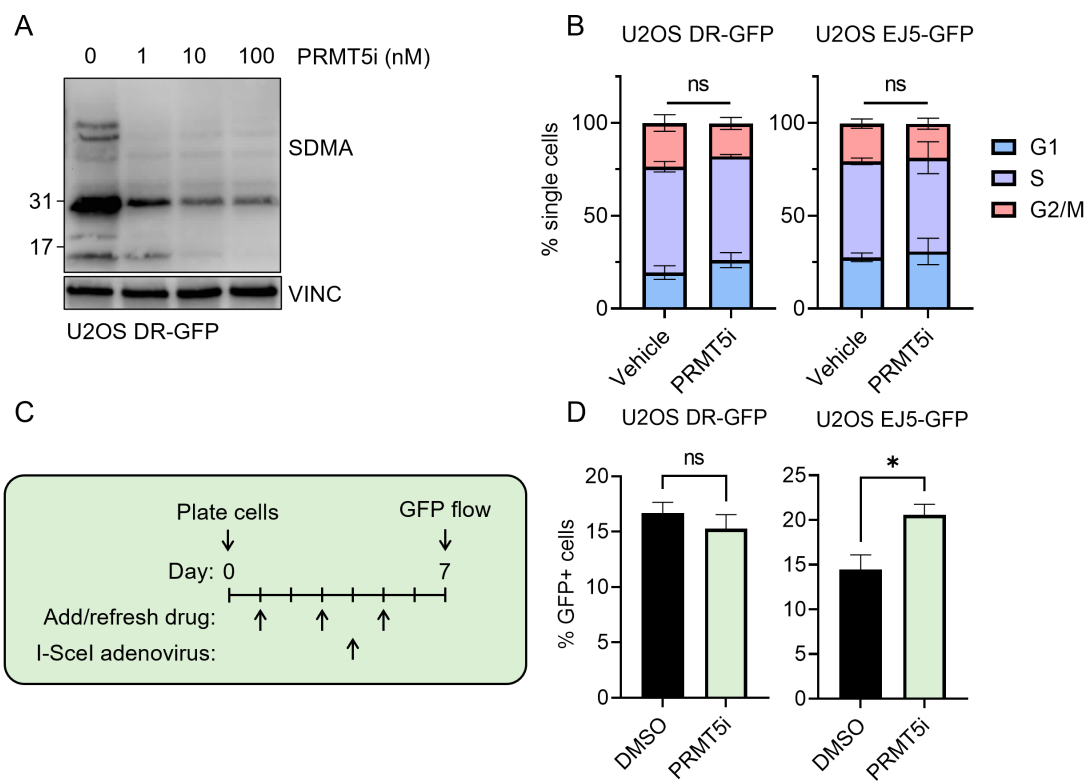


Figure 5.3: PRMT5 inhibitor increases DSB repair by NHEJ. **A.** Immunoblot of U2OS cells (DR-GFP) treated with increasing doses of PRMT5i for 4 days. **B.** Cell cycle distribution of U2OS cell lines treated with 0.1% DMSO or 10 nM PRMT5i for 4 days. N=3. **C.** Experimental design of reporter assay with PRMT5i pre-treatment. **D.** Results of GFP reporter assay from C. as measured by flow cytometry. N=3.

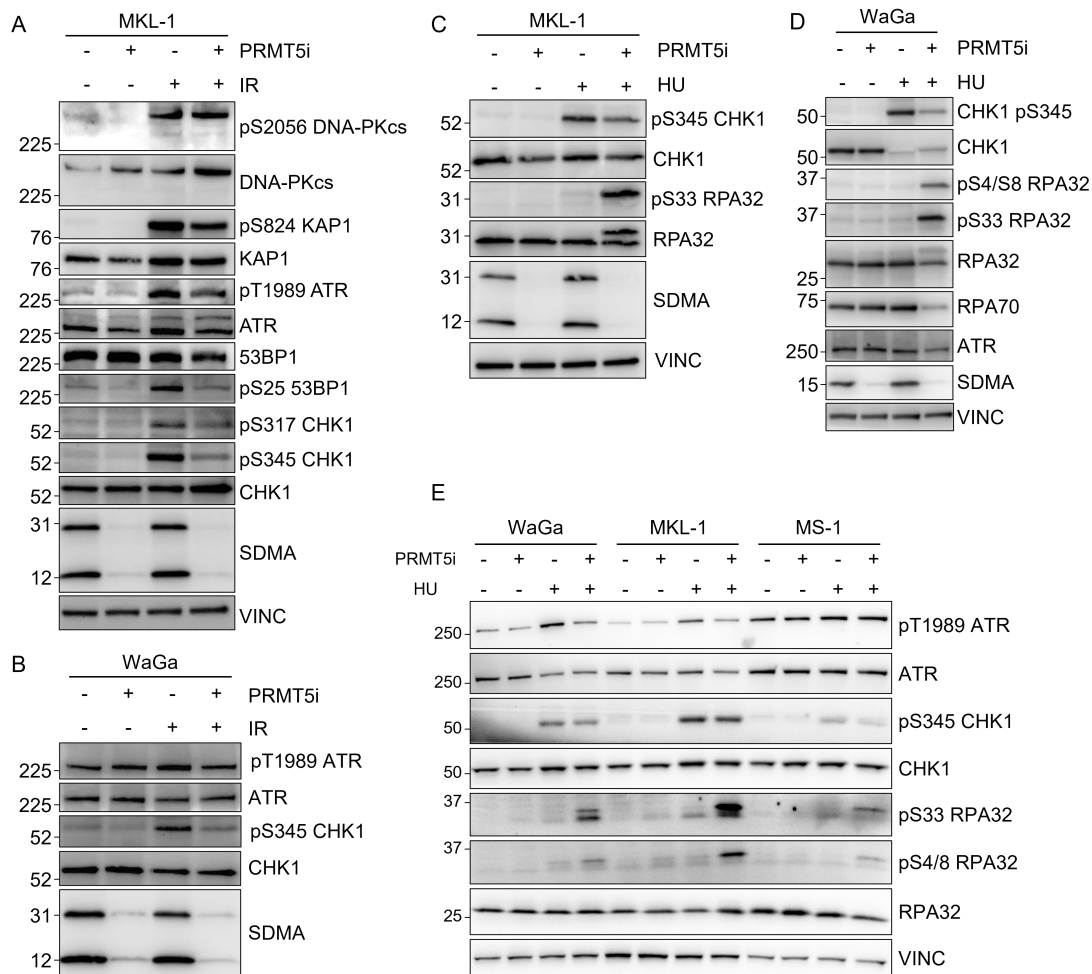


Figure 5.4: PRMT5 is required for ATR/CHK1 activation in response to extrinsic DNA damage. A-B. Immunoblot of MKL-1 (A.) and WaGa (B.) cells treated with 10 nM PRMT5i or 0.1% DMSO for 4 days and collected 1 hour post irradiation (IR) with 5 gy where indicated. N=3 for MKL-1 and N=2 for WaGa. **C-D.** Immunoblot of MKL-1 (C.) and WaGa (D.) cells pre-treated with 10 nM PRMT5i or 0.1% DMSO for 3 days prior to treatment with 500 μ M hydroxyurea (HU) for 1 (MKL-1) or 2 (WaGa) days. N=3 for MKL-1 and N=2 for WaGa.

PRMT5 loss. Interestingly, we saw that RPA32 was hyperphosphorylated in response to the combination treatment. Serine 33 of RPA32 is thought to be an ATR-specific site as discussed above, but it is a PIKK-consensus site and theoretically could also be phosphorylated by ATM or DNA-PKcs.

We asked whether this ATR/CHK1 activation defect was also observed in MS-1 cells as these cells are more resistant to PRMT5i (Figure 5.4E). We saw that ATR phosphorylation levels were higher at baseline in MS-1 cells compared to WaGa and MKL-1 and that treatment with HU or PRMT5i had no discernible effect on ATR phosphorylation. CHK1 was only weakly phosphorylated with HU treatment in MS-1 cells but even this slight activation was dampened with PRMT5i. We were able to detect RPA32 hyperphosphorylation in MS-1 cells treated with the combination of HU and PRMT5i although to a lesser extent than what we observed in WaGa and MKL-1 cells. These findings suggest that MS-1 cells are less responsive to replication stress induced by HU, but the role of PRMT5 in regulating the ATR/CHK1 response is somewhat conserved even in PRMT5i-resistant lines.

RPA32 is thought to be phosphorylated first by ATR at S33 and then by DNA-PKcs at S4 and S8 while bound to ssDNA and is often used as a marker for replication stress. Higher levels of RPA32 phosphorylation are correlated with higher levels of ssDNA, so our data suggests that PRMT5i treatment exacerbates HU-induced replication stress. However, given that RPA32 S33 is considered to be an ATR-specific site and we see high levels of RPA32 phosphorylation with low levels of CHK1 phosphorylation, it is not clear whether PRMT5 prevents ATR activation of CHK1 specifically or if PRMT5i-mediated ATR inhibition leads to hyperphosphorylation of RPA32 by another kinase. To address this question, we first compared PRMT5i treatment to treatment with an ATR inhibitor (berzosertib) in MKL-1 and WaGa cells (Figure 5.5). We

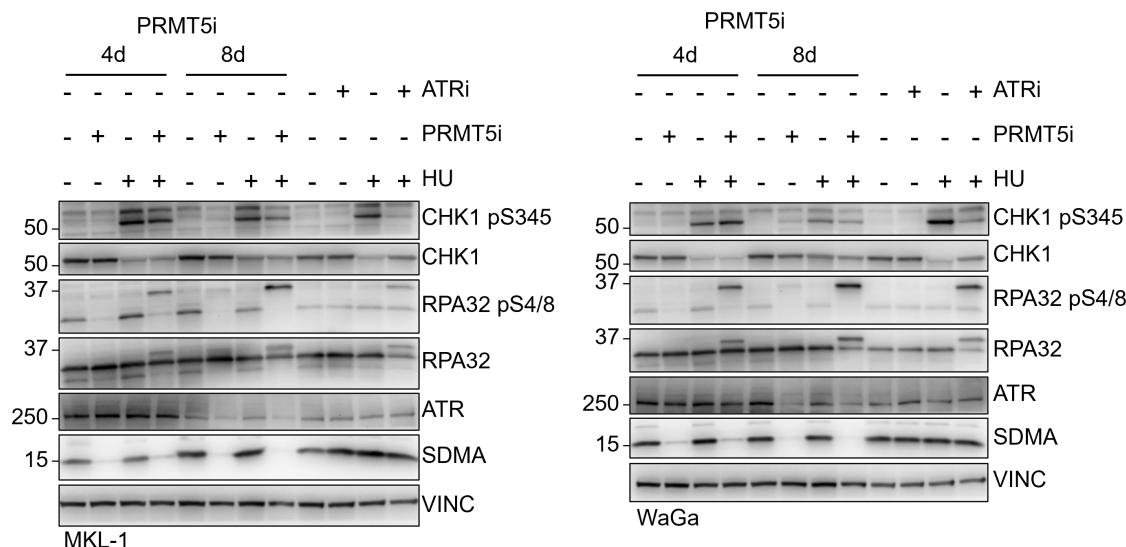


Figure 5.5: PRMT5 inhibition mirrors ATR inhibition in response to hydroxyurea-induced replication stress. Immunoblot of MKL-1 (left) and WaGa (right) cells treated with 10 nM PRMT5i for 4 or 8 days or 250 nM berzosertib (ATRi) for 24 hrs combined with 24 hr treatment of 500 uM hydroxyurea (HU). Control cells were treated with 0.1% DMSO.

saw that PRMT5i reduced CHK1 phosphorylation similarly to berzosertib treatment in both cell lines, especially at the later time point. We also saw that RPA32 was hyperphosphorylated not only with PRMT5i treatment in combination with HU but also with ATRi combined with HU. When we blotted for ATR, we saw that extended treatment with PRMT5i led to reduced levels of total ATR with PRMT5i treatment in both cell lines, suggesting that PRMT5 regulates ATR expression. Altogether these findings reinforce the conclusion that PRMT5 is required for ATR activity.

Although hyperphosphorylation of RPA32 is thought to depend on ATR activity, we've shown that RPA32 can be hyperphosphorylated in response to HU even when ATR is inhibited directly with an ATR inhibitor or indirectly through PRMT5 inhibition. In order to determine if another PIKK is responsible for RPA32 phosphorylation

with PRMT5i treatment, we decided to treat MKL-1 and WaGa cells with PRMT5i and HU in combination with different PIKK inhibitors. To do that, we first treated WaGa cells with an ATM inhibitor, an ATR inhibitor, or a DNA-PK inhibitor in combination with either HU or IR to identify an effective dose for each inhibitor in our system (Figure 5.6). We used KU-55933 as an ATM inhibitor (ATMi), berzosertib as an ATR inhibitor (ATRi), and AZD7648 as a DNA-PKcs inhibitor (DNAPKi). We confirmed that each inhibitor was working as expected at the dose and time point we chose: DNAPKi reduced DNA-PKcs phosphorylation, ATRi reduced ATR phosphorylation, and ATMi reduced ATM phosphorylation. ATRi reduced CHK1 phosphorylation while ATMi reduced both KAP1 and CHK2 phosphorylation in response to IR as expected. γ -H2AX levels were reduced by both ATMi and DNAPKi in combination with HU treatment. Interestingly, we noticed that RPA32 was only hyperphosphorylated as S33 and S4/8 with the combination of HU and ATRi; no other PIKK inhibitor had the same effect when combined with HU or IR.

Using these doses, we then combined these PIKK inhibitors with PRMT5i and HU (Figure 5.7). We pre-treated with PRMT5i for 3 days prior to treating with PRMT5i, HU, and a PIKKi for an additional 1 day. We chose this treatment strategy because the effect of PRMT5 inhibitors on cell viability is time dependent on the scale of days while PIKK inhibitors take effect very quickly. We saw that ATM and ATR phosphorylation was reduced with PRMT5i treatment compared to DMSO in all conditions we tested, and total ATM levels were reduced with PRMT5i treatment as well. DNA-PKcs phosphorylation slightly increased with PRMT5i treatment alone, in combination with HU, or in combination with HU and ATMi compared to DMSO. ATRi alone increased DNA-PKcs phosphorylation suggesting that DNA-PKcs is activated when ATR is inhibited during replication stress. When we examined the effect on

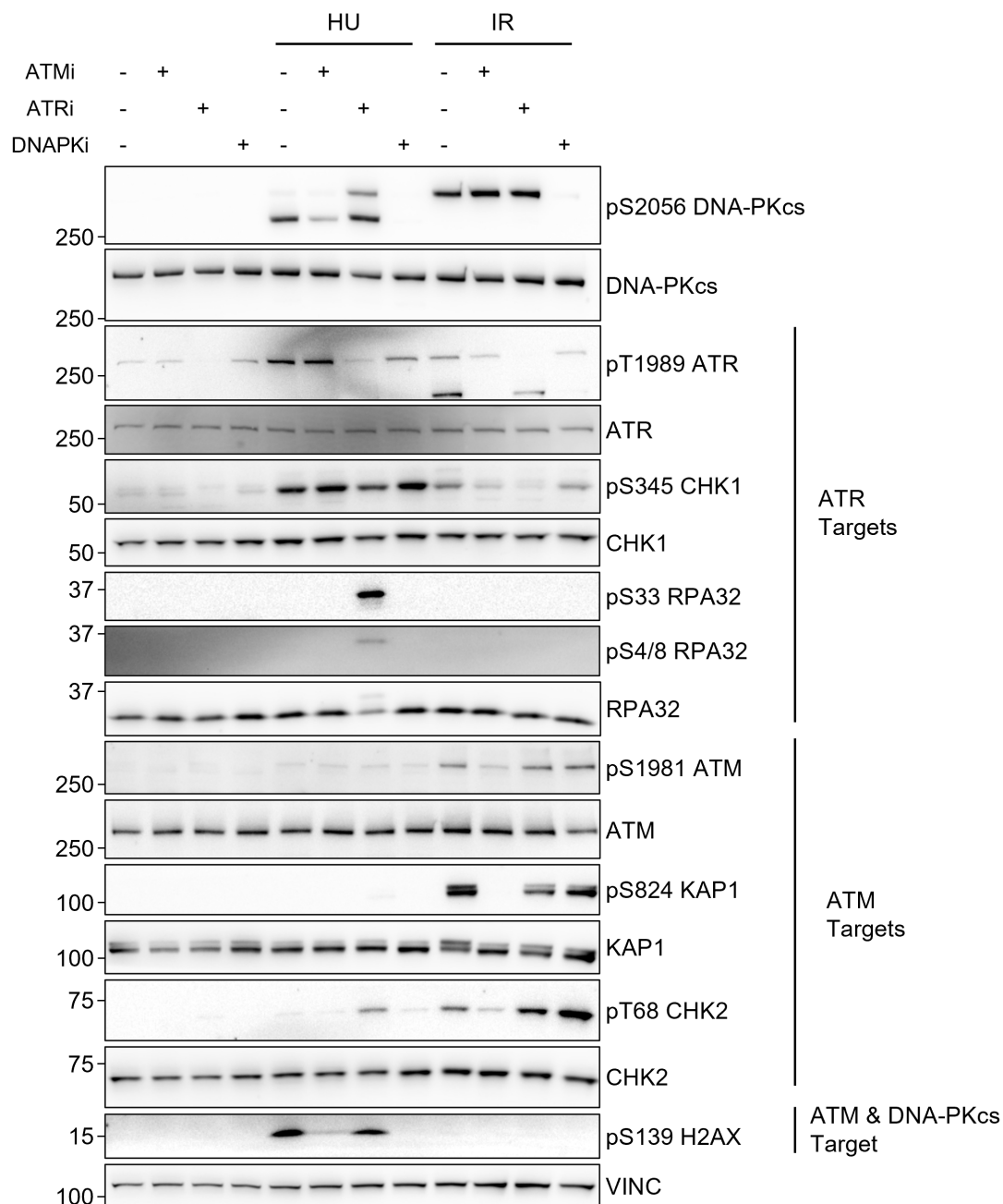


Figure 5.6: DNA damage response to replication stress and double stranded breaks in WaGa cells. Immunoblot of WaGa cells treated with hydroxyurea (HU) or irradiation (IR) in combination with PIKK inhibitors or a 0.1% DMSO control. HU = 24 hr with 500 uM HU; IR = 1 hour post irradiation with 5 gy; ATMi = 24 hr with 10 uM KU55933; ATRi = 24 hr with 250 nM berzosertib; DNA-PKi = 24 hr with 10 uM AZD7648.

RPA32 phosphorylation, we again saw that PRMT5i in combination with HU led to hyperphosphorylation of RPA32, and ATMi had no effect on RPA32 phosphorylation. ATRi with HU led to an even greater increase in RPA32 hyperphosphorylation independently of PRMT5i treatment. Treatment with DNAPKi completely abolished RPA32 phosphorylation, suggesting that DNA-PKcs regulates RPA32 phosphorylation with PRMT5i treatment during replication stress. CHK1 phosphorylation was reduced with PRMT5i in combination with HU compared to DMSO while CHK2 phosphorylation seemed to increase. Notably, CHK2 phosphorylation was decreased with ATMi combined with HU compared to HU alone but then increased with the addition of PRMT5i and was totally abolished with DNAPKi. These findings suggest that DNA-PKcs can regulate both CHK2 and RPA32 phosphorylation during replication stress with PRMT5 inhibition.

5.5 DISCUSSION

In this chapter, we investigated the role of PRMT5 in regulating the DSB response, particularly in the context of replication stress. We first showed that PRMT5i treatment alone induces replication-associated DSBs in sensitive MCCP cell lines as measured by γ -H2AX signal. Here we used both immunoblotting and flow cytometry based assays to measure total γ -H2AX levels. An alternative strategy that could be used to strengthen these findings is immunofluorescence as this technique allows for the distinction between total γ -H2AX levels and γ -H2AX puncta that are more specific to DSBs. The identification of elevated levels of replication-associated γ -H2AX supports a model where PRMT5 is required for genome stability, and PRMT5i treatment leads to the accumulation of DNA damage that eventually leads to cell death.

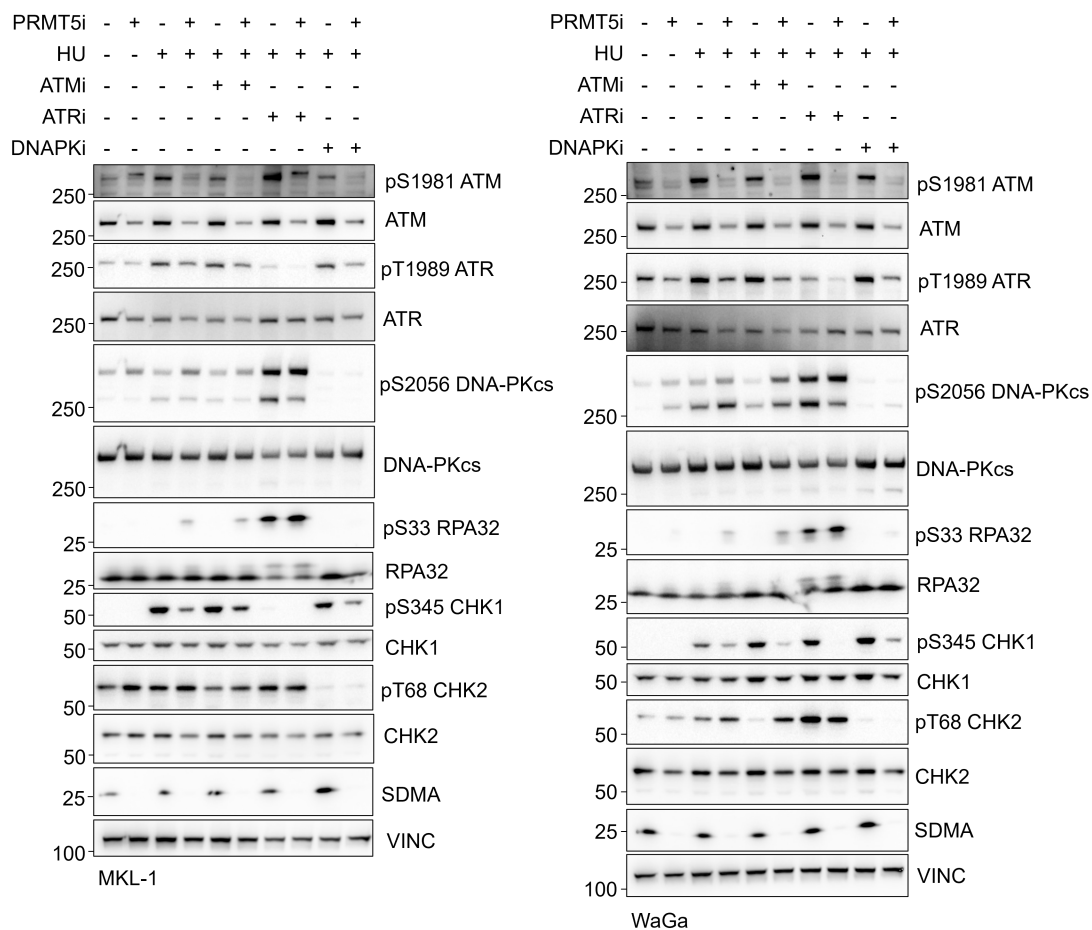


Figure 5.7: DNA-PKcs phosphorylates ATM and ATR targets with PRMT5i treatment. Immunoblot of MKL-1 (left) and WaGa (right) cells treated with PRMT5i, hydroxyurea (HU), and PIKK inhibitors or a 0.2% DMSO control. PRMT5i = 4 days with 10 nM PRMT5i; HU = 24 hr with 500 uM HU; ATMi = 24 hr with 10 uM KU55933; ATRi = 24 hr with 250 nM berzosertib; DNA-PKi = 10 uM AZD7648.

There are at least two models in the field to explain how PRMT5 inhibition could cause DNA damage, and both models center around the idea that PRMT5 is required for the response to intrinsic DNA damage, particularly during DNA replication. One model posits that PRMT5 regulates expression and/or activity of proteins involved in R loop resolution such as DDX5 or SMN^{199,200,219}. Loss of PRMT5 therefore leads to an increase in R loops which can cause fork stalling and fork collapse ultimately resulting in DSBs during DNA replication. Although we did not test this hypothesis directly, we did see that a number of previously reported factors involved in R loop resolution (reviewed in²²⁰) such as the RNA helicase AQR and a component of the THO/TREX complex THOC3 were negatively selected in our MKL-1 CRISPR screen from Chapter 4 (Supplemental Table A.1). Additional experiments are necessary to determine whether PRMT5i increases R loop formation in PRMT5i-sensitive lines and whether this correlates with sensitivity.

Another explanation for how PRMT5 loss increases DNA damage is that PRMT5 regulates the DDR and without proper DDR signaling, unresolved DNA damage accumulates. One source of potential DNA damage in a normal, unstressed dividing cell comes from DNA lesions. DNA lesions are commonly encountered by DNA polymerases with a steady state of about 10,000 lesions per cell in unstressed cells (reviewed in²¹⁷). When a replication fork encounters a lesion, the fork will stall and if not resolved fork stalling can lead to DNA breaks. ATR/CHK1 signaling and the HR repair response are thought to be particularly important for resolving this replication stress before the cell enters into mitosis and undergoes cell division. We found that PRMT5 inhibition mimicked ATR inhibition in response to HU-induced replication stress in a number of ways: not only were ATR and CHK1 phosphorylation levels reduced but RPA32 was hyperphosphorylated with both combination treatments across

cell lines. MS-1 cells appeared to be less responsive to replication stress induced by HU overall as measured by no increase in ATR phosphorylation and low levels of CHK1 phosphorylation. We did see however that PRMT5i treatment slightly reduced CHK1 phosphorylation levels and led to hyperphosphorylation of RPA32, albeit to a lesser extent than in MKL-1 or WaGa.

This finding was particularly interesting as RPA32 hyperphosphorylation is thought to be dependent on ATR activity. Using combination inhibitor treatments, we showed that when PRMT5 is inhibited, DNA-PKcs is necessary for RPA32 hyperphosphorylation. Given that the S33 site is a consensus PIKK site, we predict that DNA-PKcs directly phosphorylates RPA32 during replication stress when ATR is inhibited. Overall, our data show that PRMT5i induces replication-associated DNA damage and interferes with the ability of the cell to respond to this damage by reducing ATR/CHK1 activation. Further work is necessary to determine whether the loss of ATR/CHK1 signaling contributes to elevated levels of replication-associated DSBs observed with PRMT5i and to identify a mechanism of ATR-mediated regulation by PRMT5.

6

Discussion and Conclusions

In this dissertation, we investigated molecular mechanisms contributing to the development of Merkel cell carcinoma (MCC) in an effort to identify and characterize targetable oncogenic pathways contributing to this disease. Previous work has shown that investigating the activities of the virally-driven SLaP complex can lead to the identification of additional therapeutic targets in MCCP tumors^{67,63}. We studied the activities of two enzymes, the acetyltransferase TIP60 which is expected to play a critical role in gene activation by the SLaP complex, and the arginine methyltransferase

PRMT5, a downstream target of this complex. Our investigation into the oncogenic activities of TIP60 was somewhat hindered by the lack of specific methods to target TIP60 catalytic activity and we found even previously published TIP60 shRNA had low efficacy or off-target effects.

By contrast, multiple drug discovery efforts have led to the identification of many PRMT5-specific inhibitors, several of which have entered into clinical trials. We focused our efforts on characterizing the response of MCC cells to PRMT5 inhibition and investigated the molecular functions of PRMT5 that contribute to MCC growth. We found that both MCCP and MCCN cells are highly sensitive to PRMT5 inhibition in a p53-independent manner. We used genome-wide approaches to characterize the effect of the PRMT5 inhibitor on biological processes and found evidence indicating that PRMT5 promotes MCC growth through its role in regulating mRNA splicing. We discovered that of the genes most affected by PRMT5 splicing regulation, many were involved in DNA damage repair. Further investigation into the effect of PRMT5 on the DNA damage response revealed that PRMT5 is required for genome stability and ATR activation in sensitive MCC cells. In comparison, naturally resistant MCC cells had higher levels of ATR at baseline and lower levels of replication-associated double strand breaks with PRMT5 inhibitor. Overall, our data demonstrated that PRMT5 is a promising therapeutic target in MCC and independently identified mRNA splicing regulation of the DNA damage response as a key feature of PRMT5 oncogenic activities. In this chapter, we will detail the major findings and conclusions of our work, discussing potential mechanisms to explain our results, and highlighting opportunities for additional mechanistic investigation in future studies.

6.1 CHARACTERIZING THE MECHANISM OF THE GENE ACTIVATION BY THE SLAP COMPLEX REVEALS ADDITIONAL LAYERS OF COMPLEXITY.

In this dissertation, we first examined the mechanisms of gene transactivation by the SLaP complex and showed that TIP60 (*KAT5*), the acetyltransferase subunit of the complex, is expressed in multiple isoforms that are splice variants of one another. By taking an isoform-aware approach, we found that all previously-annotated TIP60 isoforms were expressed in MCC and co-precipitated with the SLaP complex. All four TIP60 isoforms contain the chromodomain and the MYST acetyltransferase domain and are predicted to have catalytic activity suggesting that multiple isoforms could contribute to gene transactivation by SLaP.

To interrogate the functional role of TIP60 broadly in MCC growth and more specifically in histone acetylation and gene transactivation by SLaP, we designed an approach to target TIP60 expression using shRNA and then rescue expression with specific TIP60 isoforms using both wild-type and catalytically-dead TIP60 constructs. We tested a number of TIP60 shRNA, including many that were previously published or were designed by the Broad Genetic Perturbation Platform (GPP), and identified two TIP60-specific shRNA that targeted all 4 TIP60 isoforms and reduced total TIP60 expression. Using these shRNA, we found that knockdown of TIP60 led to reduced MCC cell viability and reduced levels of histone acetylation of H2A.Z (H2A.Zac) and H4 at lysine 16 (H4K16). Overexpression of TIP60 α and TIP60 β , individually or in combination, did not rescue either the cell viability defect or the histone acetylation loss however. The most straight-forward explanation for this finding is that both of the shRNA we tested had off-target effects although it is possible that all four TIP60 isoforms are required for rescue. Despite seeing similar levels of TIP60 knockdown

between the two shRNA we used, we noticed that total H2A.Z levels were reduced with only one of the shRNA which may be further evidence of off-target effects.

Interestingly, we found that overexpression of TIP60 α alone or in combination with TIP60 β also reduced total H2A.Z levels when compared with a GFP control. There are a number of different possibilities that could explain this phenomenon. One possibility is that TIP60 α overexpression activates a transcriptional repressor, possibly through the transcriptional activities of the SLaP complex, which then inhibits transcription of *H2AZ1*. This hypothesis assumes that overexpression of TIP60 α increases expression of SLaP target genes, an assumption that has not been experimentally tested. An alternative theory is that H2A.Z expression is regulated directly or indirectly by a non-histone target of TIP60 such as p53^{221,222}. Although *H2AZ1* is not considered a p53 target gene according to the data sets in Target Gene Reg²²³, it is considered to be a G2/M gene, meaning its expression is activated in late cell cycle. Additionally, *H2AZ1* was identified as a target of the DREAM complex, a transcriptional repressor complex thought to mediate gene repression during G0 and coordinate gene silencing throughout the cell cycle²²⁴. If TIP60 overexpression alters the cell cycle distribution of MKL-1 cells, it could explain the observed reduction in total H2A.Z levels.

Although we weren't able to conclusively show that TIP60 acetylates H2A.Z from our data alone, other groups have reported that H2A.Z is a TIP60 acetylation target^{123,124}. H2A.Z is also reportedly deposited in chromatin by P400¹²⁸, another subunit of SLaP. We found by ChIP-seq that H2A.Z and H2A.Zac signal was enriched at specifically at gene promoters bound by the SLaP complex. These findings are in alignment with our model that the TIP60/P400 complex promotes gene activation at L-MYC target genes through H2A.Z deposition and acetylation.

6.1.1 IMPORTANT CONSIDERATIONS FOR shRNA-BASED GENE KNOCKDOWN.

An unexpected finding of our efforts to characterize the role of TIP60 in histone acetylation and gene transactivation in MCC was that a number of previously published or highly computationally-scored TIP60-specific shRNA were ineffective or had non-specific effects. Using two previously published TIP60 shRNA (Simpson et al. 2018¹⁴¹ and Zhao et al. 2018⁶²), we found that neither had an effect on TIP60 expression as measured by immunoblot in our system. We also used the GPP by the Broad Institute to identify shRNA computationally predicted to be effective and specific. The GPP reports an intrinsic score for shRNA based on predicted clonability and knockdown efficiency as well as an adjusted score that factors predicted specificity into the intrinsic score. Two of the shRNA we tested (TRCN0000293417 and TRCN0000293476) had high adjusted scores (>15). When we tested these experimentally, only one had an effect of TIP60 expression. We also used two shRNA with lower adjusted scores (<7) and found that one out of the two reduced TIP60 expression. Although computationally predicted shRNA scores can be a useful metric for prioritizing shRNA sequences to test, these results highlight the importance of testing a wide range of shRNA experimentally to identify those with high knockdown efficiency in the target system.

After testing 9 TIP60 shRNA, we identified 2 (shTIP60-1 and shTIP60-6) that efficiently knocked down all TIP60 isoforms and 7 that had no measurable effect on TIP60 expression. We were unable to rescue the effects of these shRNA on histone acetylation or cell viability despite robust constitutive expression of the major TIP60 isoforms so it's possible that both shRNA had significant off target effects. When looking for possible sources of off-target effects, we found that the guide sequence of shTIP60-1 targeted a region of the *KAT5* gene that overlapped with the *RNASEH2C*

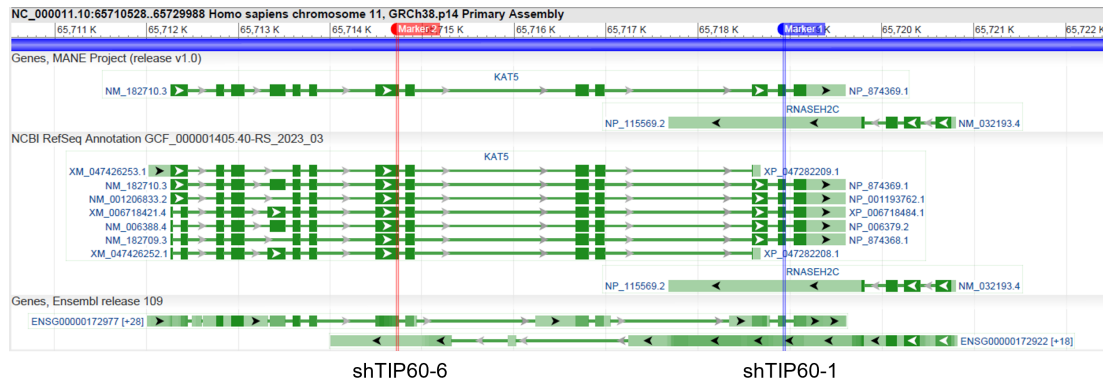


Figure 6.1: The *KAT5* gene overlaps with *RNASEH2C* on the opposite strand. Genomic region surrounding the *KAT5* gene from the National Center for Biotechnology Information gene browser. The sequences used to target TIP60 for knockdown are highlighted and labeled.

gene which is encoded on the negative strand (Figure 6.1). The shTIP60-6 sequence did not overlap with canonical coding sequence of *RNASEH2C* but did overlap with a previously annotated *RNASEH2C* transcript. It's possible that the passenger strand of these shRNA, the sequence complementary to the target shRNA sequence, affects expression of *RNASEH2C* although this has not been tested experimentally.

Previous work found that the passenger shRNA sequence can cause off-target effects and a few strategies have been identified to mitigate these effects¹⁴⁴. These include designing shRNA with shorter target sequences (18 nts) and shorter loops (<5 nts) to favor specific shRNA processing pathways that degrade the passenger strand¹⁴⁴. The Broad GPP database (using rule set 9) uses 21-mer shRNA sequences and only has a restriction on a minimum loop size with no restriction on maximum loop size. Based on these findings, we recommend that future efforts to knock down TIP60 take into consideration both target sequence length and loop size, design shRNA targeting the 5' end of *KAT5* that does not overlap with *RNASEH2C*, or consider alternative methods for gene knock down.

6.2 UNDERSTANDING THE MOLECULAR TARGETS OF THE SLAP COMPLEX CAN LEAD TO NOVEL THERAPEUTIC STRATEGIES FOR MCC TUMORS.

Understanding the contribution of TIP60 to SLAP mediated gene activation was of interest to us because the downstream targets of SLAP have proven to be important for MCC growth. For example, MDM2 and CK1 α were two SLAP targets that were shown to be essential for MCC viability⁶⁷. MDM2 and CK1 α are both involved in regulating p53 expression, and investigation into these SLAP targets found that MDM2 inhibitors combined with CK1 α degraders synergized to kill MCC cells through p53 activation in both *in vitro* and *in vivo* models of p53 wild-type (WT) MCC⁶⁷. Another example is LSD1, RCOR2, and INSM1, three proteins involved in the LSD1-CoREST transcriptional repressor complex⁶³. Park et al. showed that targeting LSD1 pharmacologically induced a neuronal differentiation program that led to cell growth arrest⁶³ which was independently validated⁶⁴. These studies highlight the role of the SLAP complex in activating critical molecular pathways that contribute to MCC growth and development.

We re-examined the list of SLAP target genes and identified a number of arginine methyltransferases including PRMT1, 3, 5, and 7. PRMT5 in particular has been implicated in the development of a variety of cancers and has been the subject of many drug discovery efforts (reviewed in⁸⁰). We therefore asked whether targeting PRMT5 using small molecule inhibitors could be a viable therapeutic strategy in MCC. A number of inhibitors have been designed to target the substrate binding pocket of PRMT5 (Figure 6.2: H4 peptide binding site) including EPZ-015666 (GSK-3235025) and GSK-3326595. Others like JNJ-64619178 have been designed to target the S-adenosyl methionin (SAM) binding site to block methylation activity (Figure 6.2). In

this dissertation, we treated a panel of MCCP cell lines with JNJ-64619178 and GSK-3326595. We determined that both inhibitors had similar effects on cell viability with GI50 concentrations in the sub-nanomolar to nanomolar range, but JNJ-64619178 was about 250 times more potent. Out of six MCCP cell lines, four were highly sensitive and two were resistant up to 100 nM of JNJ-64619178. We therefore treated a panel of MCCN cells with JNJ-64619178 (PRMT5i) and found that the established MCCN cell lines were also highly sensitive to PRMT5i. We treated MCC patient-derived cell lines (PDCLs) and again found GI50 concentrations in the nanomolar range.

PRMT5 is reportedly a common essential gene according to data from the Dependency Map portal by the Broad Institute (<https://depmap.org/portal/gene/KAT5?tab=overview>) however our data and previous work has found that sensitivity to PRMT5 inhibition can vary greatly between cell lines⁷⁵. An interesting question that we did not address here is whether the effect of PRMT5 knockout is similar to the effect of PRMT5 inhibitor treatment on MCC viability. It's possible that our PRMT5i-resistant lines, MKL-2 or MS-1 cells, would tolerate a partial or complete loss of PRMT5 expression while MKL-1 or WaGa cells would not. This hypothesis could be tested experimentally using a CRISPR Cas9 targeted gene editing technique to mutate the PRMT5 gene. Using a genetic approach in addition to a pharmacological approach would strengthen confidence in our findings.

Another important remaining question is how sensitivity to PRMT5 inhibitor compares between MCC tumors and normal tissue. Investigation into this question by treating MCC cells side-by-side with normal skin cells like keratinocytes would allow us to determine whether there is a dose range at which PRMT5 inhibitor treatment would effectively inhibit MCC growth without causing severe toxicity to normal cells. A previously identified indicator of heightened sensitivity to PRMT5 inhibi-

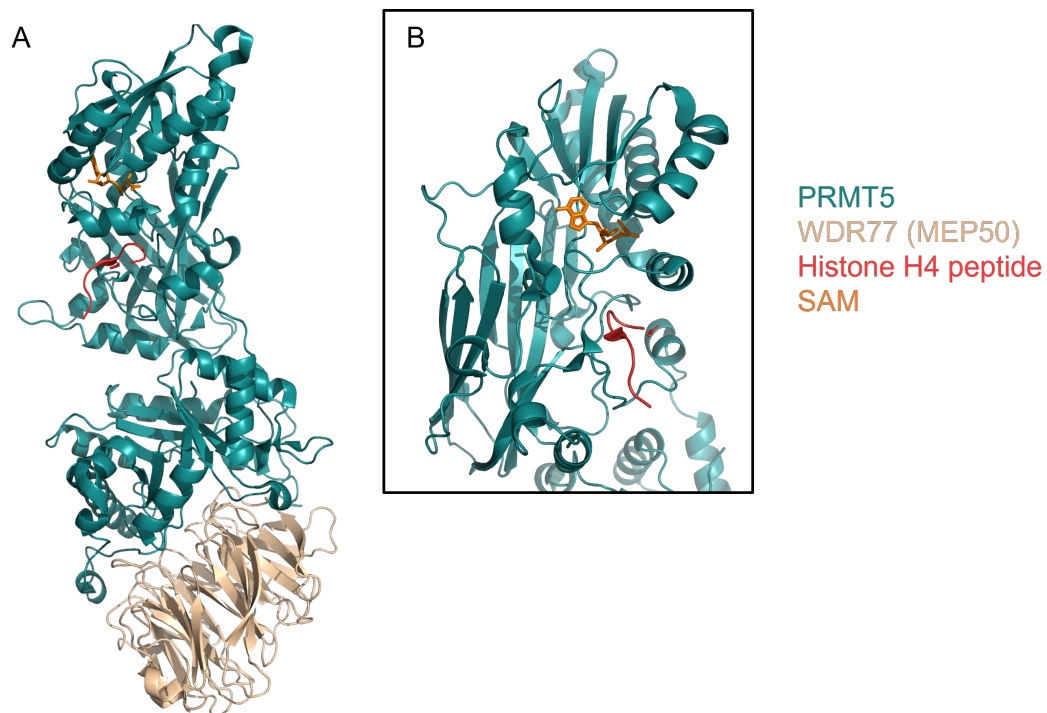


Figure 6.2: Structure of PRMT5 bound to WDR77. Crystal structure from Antonysamy et al.²²⁵ of one subunit of PRMT5 bound to WDR77 (MEP50), a histone H4 peptide, and the SAM methyl donor. PRMT5 was colored in deep teal, WDR77 in wheat, the H4 peptide in red, and the SAM molecule in orange using PyMOL⁴⁷. PDB=4GQB. **A.** View of the entire PRMT5:WDR77 structure. **B.** A close-up view of the PRMT5 substrate and SAM binding pockets.

tion is MTAP deletion^{226,227}. Loss of MTAP (methylthioadenosine phosphorylase) leads to the accumulation of its substrate MTA (methylthioadenosine) which acts as a competitor of SAM, the methyl donor for PRMT5 and therefore inhibits PRMT5 activity. By analyzing the oncopanel sequencing data from Starrett et al.⁵⁴, we can see that MTAP is mutated (typically a shallow or deep deletion) in 16% of all MCC tumors, more commonly in those with a high tumor mutation burden (about 40%). It is also possible that the MTAP gene is epigenetically silenced in MCC which could be contributing to PRMT5 inhibitor sensitivity, and it would be useful to examine the expression levels of MTAP across PRMT5i sensitive and resistant cell lines. Additionally, small molecules that promote the MTA-PRMT5 interaction have recently been developed to specifically target PRMT5 activity in MTAP-null cancers with reduced toxicity in normal, MTAP WT cells²²⁸. Further experiments are necessary to determine whether MTAP gene status or protein expression is correlated with PRMT5i sensitivity in MCC and whether these novel MTA-specific PRMT5 inhibitors could be effective in MTAP-null MCC tumors.

6.2.1 MULTIPLE AVENUES OF P53 ACTIVITY REGULATION BY THE SLAP COMPLEX

After demonstrating that many MCC cell lines are sensitive to PRMT5 inhibition, we wanted to understand the mechanisms that contribute to PRMT5i sensitivity. Previous work has shown that PRMT5 regulates the p53 response either through direct methylation or through alternative splicing of MDM4, a negative regulator of p53^{74,75,76,77,110} which was of particular interest to us as there are many cases of p53 WT MCCP tumors. MDM4 is activated by CK1 α and can inhibit p53 through direct binding or can form a complex with MDM2 to ubiquitinate p53, targeting it for degradation (reviewed in⁶⁹). In MCC, targeting MDM2 alone or in combination with MDM4 or

CK1 α inhibitors has been shown to reactivate p53 in p53 WT MCC cells leading to p53-dependent apoptosis^{67,73}. We hypothesized therefore that PRMT5i sensitivity would be dependent on p53 activation.

Previous work has found that p53 status correlates with PRMT5i sensitivity⁷⁵, and although this was not the case in the four MCCN lines we tested, we found this to be true in MCCC cells: the two p53 mutant MCCC lines we tested were both resistant to PRMT5i treatment compared with the four p53 WT MCCC lines. We showed that in p53 WT lines, PRMT5i treatment led to MDM4 loss and p53 activation as measured by increased p53 and p21 expression in alignment with the theory that PRMT5 regulates p53 activity through MDM4. Notably, PRMT5 has been shown to regulate p21 expression through a number of p53-independent mechanisms. Saha et al. and Zhang et al. both found that PRMT5 silences p21 epigenetically through histone arginine methylation^{154,155} and PRMT5 inhibition led to activation of p21 in a p53-independent manner. Li et al. also found that PRMT5 inhibition induces p53-independent p21 expression and argue that this occurs indirectly as a result of PRMT5 methylation of TRIM21 which then regulates p21 expression¹⁵⁶. To address these alternative hypotheses, we treated a p53 knockout cell line with PRMT5i and showed that p53 loss reduced p21 to background levels, indicating that PRMT5 regulation of p21 is entirely p53-dependent in our system.

P53 activation and p21 expression is associated with cell cycle arrest, so we asked whether PRMT5i altered the cell cycle distribution of our cells. We found that the cell cycle distribution of both WaGa and MKL-1 was affected, albeit in different ways. WaGa cells began accumulating in G1 as early as two days with PRMT5i treatment while MKL-1 cells showed no signs of cell cycle arrest until day 8 at which point there was a significant enrichment for cells in G2/M. Both types of cell cycle arrest

have been previously reported in cells treated with PRMT5i. Gerhart et al. reported that the most sensitive mantle cell lymphoma (MCL) lines they tested underwent a G1 arrest with PRMT5 inhibition whereas there was no effect on cell cycle for less sensitive MCL lines⁷⁵. Additionally, in breast cancer lines they found divergent phenotypes between two similarly sensitive lines: MCF-7 cells accumulated in G1 after 2 days of treatment while MDA-MB-468 cells saw an increase in 4N and >4N populations after 7 days of treatment. Gerhart et al. note that they only saw a G1 arrest in the p53 WT lines they tested, however we found that WaGa cells accumulated in G1 even with expression of a dominant negative p53 mutant, indicating that this arrest is p53-independent.

Besides p53, there are a number of possible mechanisms through which PRMT5 could induce a G1 cell cycle arrest. PRMT5 has previously been implicated in regulating E2F1 activity to promote cell cycle progression through G1^{112,158,159}, with loss of E2F1 methylation associated with a G1 arrest and apoptosis. Additional work is necessary to determine whether an E2F1-driven mechanism could explain the cell line-specific G1 cell cycle arrest with PRMT5i treatment in MCC.

P53 activation can also trigger apoptosis, so we asked whether PRMT5i induced apoptosis using an annexin V staining assay. We found that in WaGa cells, PRMT5i treatment caused an increase in the proportion of apoptotic cells after 8 days of treatment. However p53DD expression only slightly increased the proportion of live cells at each time point, suggesting that PRMT5i induced apoptosis in a primarily p53-independent manner.

These data, in combination with our cell viability data showing that loss or inactivation of p53 in p53 WT MCC cells had no effect on cell viability with PRMT5i treatment, confirmed that p53 activation is not required for PRMT5i sensitivity. We

hypothesized however that because PRMT5 reduced total MDM4 levels and activated p53 that PRMT5i would synergize with MDM2 inhibition. We treated MKL-1 and WaGa cells with increasing doses of both nutlin-3A and PRMT5i at two time points and found that the combination varied between an additive to a synergistic effect, particularly at later time points. We conclude that although p53 activation is not essential for PRMT5i-induced apoptosis, PRMT5i treatment increases sensitivity to p53 activation through MDM2 inhibition. Further work is necessary to determine whether this finding extends to CK1 α inhibitors as well.

6.3 PRMT5 REGULATION OF THE SPLICEOSOME IS CRITICAL TO MCC GROWTH.

PRMT5 activity has been implicated in many diverse cellular processes making it challenging to identify which PRMT5 functions are critical in specific cellular contexts. Although p53 was shown to be an important contributor to PRMT5 inhibitor sensitivity in other cancer cell lines, our data clearly show that p53 activity is not required for PRMT5i sensitivity in MCC. We therefore performed a CRISPR screen using MKL-1 cells treated with a low dose of PRMT5i to identify genes that when lost contribute to PRMT5i sensitivity or resistance. Notably, loss of p53 was not highly scored in our CRISPR screen in accordance with our previous work. By using a gene ontology enrichment analysis, we determined that genes involved in mRNA processing and splicing were enriched among the negatively selected hits. These included a number of proteins that were previously determined to be direct PRMT5 targets by three different studies using mass spectrometry-based approaches^{83,90,89}. Although two out of the three known PRMT5 adaptor proteins did not meet our significance thresholds, we found that CLNS1A scored negatively while COPR5 and RIOK1 scored

positively in our screen. All three adaptor proteins compete for binding to the same region of PRMT5, so loss of RIOK1 or COPR5 would likely increase association between PRMT5 and CLNS1A. We concluded therefore that although PRMT5 has many diverse targets including ribosomal proteins and histones, methylation of splicing factors is the most important role of PRMT5 that is inhibited with PRMT5 inhibitor treatment in MCC.

We therefore examined how PRMT5 inhibition altered mRNA splicing patterns in MCC. PRMT5 has been implicated previously in regulating splicing of primarily detained introns (DIs) and cassette exons (CASs). Loss of PRMT5 activity is typically associated with increased levels of DIs and increased instances of exon skipping, with some variation between studies as to whether DIs or CASs are most affected. We characterized the effect of PRMT5 inhibition in MCC by performing mRNA sequencing of both MKL-1 and WaGa cells treated with PRMT5i for 4 and 8 days. We found that the PRMT5 loss predominantly altered expression of DIs, and more than half of all DIs that we detected in MCC cells were significantly differentially expressed with PRMT5i treatment. The vast majority of differential DIs increased with PRMT5i treatment, indicating that PRMT5 regulates removal of DIs. We also found that the effect of PRMT5 on splicing between cell lines was highly conserved: over 90% of DIs that significantly increased with 4 days of treatment detected in MKL-1 cells were shared in WaGa cells. Of note, there were fewer DIs called in MKL-1 cells compared with WaGa cells which is likely due to the MKL-1 data set having a higher level of noise: our PCA plots of the total RNA expression data showed that the WaGa replicates clustered more closely than the MKL-1 replicates. It's possible that by adjusting our thresholds for significance we could see an even greater overlap between data sets.

This extremely high degree of overlap of differential DIs between cell lines indi-

cated to us that this was likely a direct effect of PRMT5 loss and that regulation of DIs by PRMT5 is highly conserved at least between MCC cells. Previous work has found that a subset of DIs are evolutionarily conserved between cell types whereas others are cell type-specific¹¹⁴. Boutz et al. found that in general, DIs were commonly found in genes involved in RNA metabolism, DNA repair, apoptosis, and cell cycle¹¹⁴ and Braun et al. found similar results for PRMT5-regulated DIs specifically, reporting that these DIs were enriched in genes involved in cell cycle progression, chromosome segregation, RNA biology, and DNA repair⁹⁹. When we performed a gene ontology enrichment analysis of the differentially upregulated DIs in our data, we saw many similar terms including DNA repair, cell cycle, and mitosis. Noticeably however one of the top enriched gene ontology terms in our data set was protein transport. This term included many genes involved in nuclear transport including a number of importins such as IPO5 and IPO7-9 as well as components of the nuclear pore complex like NUP107, NUP93, and NUP214 among many others. It would be interesting to compare the DIs identified in our data of both untreated and treated MCC cells to those previously identified in other cell types to determine which DIs are MCC-specific and which are more broadly conserved.

Based on the finding that transcripts containing DIs are retained in the nucleus and are therefore unable to be translated, a prevailing assumption in the field is that an increase in the proportion of transcripts containing DIs will lead to a decrease in expression of the protein product. However, the correlation between RNA and protein expression is reportedly quite variable, with the degree of correlation changing depending on how many genes are analyzed, the method used to measure RNA and protein levels, and the specific gene products that are included^{229,194}. Previous studies have found that 0-50% of differentially expressed proteins have concordant expression with

the corresponding mRNA, indicating that for most proteins the corresponding mRNA expression levels are discordant¹⁹⁴. Koussounadis et al. specifically examined the correlation between mRNA and protein levels before and after treatment with carboplatin and found low overall correlation between log fold change of mRNA vs protein with a Pearson correlation coefficient of $R = 0.08$. Many studies examining the relationship between mRNA and protein levels don't take RNA splicing into consideration which could be a contributing to the low levels of correlation or concordance.

To test this assumption and to better understand how PRMT5 regulates protein expression, we performed quantitative mass spectrometry experiments using MKL-1 and WaGa cells treated with PRMT5i for 4, 8, and 12 days. We were able to detect over 6,500 proteins shared in both MKL-1 and WaGa cells and found that the protein expression changes between MKL-1 and WaGa were highly correlated, with a correlation coefficient of $R = 0.7$. We saw that about half were differentially expressed ($\text{padj} < 0.05$) concordantly over the 12 day time course in both cell lines with only 5% discordantly differentially expressed. We saw that for most proteins, protein expression progressively changed over the 12 day time course with the greatest changes observed after 12 days of treatment.

We previously observed, at least in WaGa cells, that a large proportion of cells were apoptotic after 12 days of PRMT5i treatment. To minimize the impact of dead cells in our proteomic analysis, we focused on changes in protein expression after 4 days of PRMT5i treatment. We found that PRMT5i treatment led to an increase in expression of proteins involved in mRNA splicing and processing with a decrease in proteins involved in cell division. Among the upregulated mRNA processing genes, many were identified in our CRISPR screen as negatively selected with PRMT5i treatment. Based on these data, we hypothesize that the loss of PRMT5 induces splicing defects which

then trigger a response to upregulate additional mRNA splicing factors in an attempt to compensate.

The loss of cell cycle protein expression in both MKL-1 and WaGa cells is somewhat surprising, as we know that after 4 days of PRMT5i treatment MKL-1 cells appear to have a normal cell cycle distribution while WaGa cells accumulate in G1. It appears in MKL-1 cells that proteins involved in cell division begin to decrease with PRMT5i treatment prior to a measurable effect in cell cycle distribution. It's possible that PRMT5 inhibition reduces cell cycle gene expression which then forces cells to accumulate in a particular cell cycle phase. This theory is in alignment with Braun et al. who argue that loss of PRMT5 increases DIs in cell cycle genes which reduces levels of productive transcripts and ultimately protein expression⁹⁹. We did identify a number of genes involved in cell cycle and cell division as containing higher levels of DIs with PRMT5i treatment including CCNB1, AURKB, and components of the anaphase-promoting complex ANAPC1, ANAPC4, and ANAPC5. Additional work is necessary to validate these findings and confirm that increased levels of DIs correlates with a decrease in protein expression prior to changes in cell cycle distribution in both cell lines.

We also asked whether correcting for DI levels would improve the correlation between mRNA and protein expression changes. Focusing on the 4 day time points in MKL-1 cells, we found that total mRNA log fold change values had a low correlation to protein log fold change values as expected, with a correlation coefficient of $R = 0.17$. Contrary to our hypothesis, correcting for DIs using EBSeq did not improve this correlation and in fact the correlation coefficient decreased slightly to $R = 0.11$. We decided to restrict our analysis to DNA damage genes that we identified as containing at least one DI and again found that the correlation between mRNA and protein was

very low, with a coefficient of $R = 0.029$ but improved 10-fold to a correlation of $R = 0.29$ when correcting for DIs. Although this analysis was performed on a selective data set, these data suggest that correcting for DIs could improve the mRNA-protein correlation for certain gene sets. We noticed that for a subset of genes, the correcting for DI expression led to an extremely low log fold change value (<-10). We used EBseq to approximate the effect of DIs on transcript expression, but this method of correction for DIs assumes one DI per transcript¹⁸¹. If a transcript for a gene contains more than one DI, this method will therefore over-correct. Integrating this analysis with long-read sequencing data to identify transcripts with multiple DIs could potentially improve the correlation between mRNA and protein even further. Ultimately, there are many additional mechanisms regulating protein expression besides transcript levels so there is almost certainly a limit to how well mRNA expression will ever correlate with protein expression.

6.4 SPLICEOSOME ACTIVITY IS INTERTWINED WITH THE DNA DAMAGE RESPONSE.

The study of PRMT5-mediated mRNA splicing has highlighted that the DNA damage response (DDR) is one of the pathways most likely influenced by changes in regulation of DIs. Our work along with previously published data found that DIs as a whole and specifically PRMT5-regulated DIs are enriched for genes involved in DNA damage repair^{114,99}.

The relationship between mRNA splicing and the DDR has been the subject of many studies which have shown that not only does the spliceosome regulate DDR transcript splicing but that loss of spliceosome components can more directly induce DNA damage. In 2009, a group in the Cimprich lab performed an siRNA screen with

γ -H2AX as a read out and found that factors involved in RNA splicing and processing as the most frequently identified hits in the screen²¹⁰. They ultimately concluded that for many mRNA processing factors the increase in DNA damage observed upon knockdown is due to an increase in R-loops in the genome as overexpression of RNase H, an endoribonuclease that specifically degrades R-loops, rescued the phenotype. R-loops, or RNA-DNA hybrids, can have important roles in regulating transcription by influencing transcription factor binding and chromatin accessibility (reviewed in^{220,230}). However when R-loop removal is improperly regulated, excess R-loops can not only interfere with RNA transcription but can cause replication stress during S phase by acting as a barrier to replication fork progression and increasing the likelihood of collisions between transcription and replication machinery (reviewed in^{231,232}). High levels of R-loops can lead to fork stalling which if not resolved results in fork collapse and ultimately a double strand break (DSB) (reviewed in^{204,217}).

PRMT5 loss has similarly been associated with an increase in DNA damage, and a number of groups have argued that this is due to an increase in R-loops^{219,199,200}. Interestingly, we identified a few mRNA processing factors that have been linked to R-loop removal²²⁰ in our CRISPR screen including the RNA helicase AQR and THOC3 as well as components of the exosome. AQR and THOC3 were also identified as up-regulated on the protein level in response to PRMT5i treatment for 4 days. We did not identify any of the better-studied R-loop processing factors in our CRISPR screen however, like RNaseH1 and RNaseH2 nor did we identify the R-loop processing factors that have been linked to PRMT5 activity such as DDX5 or SETX. Further work is necessary to determine whether PRMT5 induces R-loops in MCC and whether the ability of the cell to regulate R-loop removal contributes to PRMT5i sensitivity in MCC.

An alternative hypothesis to explain the higher levels of DNA damage associated with PRMT5 inhibition is that PRMT5 is required for responding to DNA damage. Without PRMT5 activity, intrinsic DNA damage accumulates ultimately resulting in p53 activation, cell cycle arrest, and cell death⁷⁶. DNA damage can occur normally during DNA replication if a stalled replication fork collapses resulting in a DSB, and thousands of DNA lesions are encountered by DNA polymerases during normal cell division (reviewed in²¹⁷). The ATR/CHK1 pathway is thought to be critical for regulating fork stalling and for responding to this type of replication stress (reviewed in²⁰⁴). One of the top negatively selected genes we identified in our CRISPR screen was claspin (*CLSPN*), a protein important for CHK1 activation by ATR. Interestingly, we also identified a number of additional hits in our screen that have been previously implicated in regulation of claspin stability. Among the top positively selected genes, we identified SKP1 and PLK1 which are both proteins that promote claspin degradation while USP29, a deubiquitinase that promotes claspin stability, was negatively selected (reviewed in²³³). Based on these data, and the previous literature implicating PRMT5 in regulating genome stability, we investigated the effect of PRMT5 on both genome stability and DDR pathway activation.

6.5 PRMT5 IS REQUIRED FOR ACTIVATION OF THE DDR IN RESPONSE TO BOTH INTRINSIC AND EXTRINSIC DNA DAMAGE.

We first investigated the effect of PRMT5 loss on DNA damage alone and found that sensitivity to PRMT5 inhibition correlated with increased levels of replication-associated γ -H2AX, a marker of DSBs. These findings are in alignment with Hamard et al. who reported an increase in γ -H2AX levels in the PRMT5 inhibitor-sensitive leukemia line

but not in a resistant line⁷⁶. From these data, we conclude that PRMT5 is required for maintenance of genome stability in MCC and propose that MCC cell sensitivity to loss of PRMT5 may be influenced by the ability of the cell to respond to or prevent this DNA damage.

Our data indicated that PRMT5 loss induces DSBs during genome replication, and previous literature has indicated both the ATR/CHK1 pathway and the homologous recombination (HR) repair pathway are important for resolving this type of damage (reviewed in²¹⁷). A number of groups have reported that PRMT5 is important for DSB repair by HR^{116,76} although another report found that PRMT5 plays roles in regulating both repair by HR and non-homologous end joining (NHEJ)¹⁹⁶. Using U2OS reporter cells, we found that PRMT5 inhibition increased repair by NHEJ while repair by HR was either unaffected or slightly decreased. These results are somewhat contradictory to the previous reports, as both Hwang et al. and Clarke et al. reported using similar reporter cell lines that PRMT5 knockdown decreased repair by HR, but Hwang et al. reported a decrease in repair by NHEJ as well^{196,116}. Despite these discrepancies, we expect that PRMT5 loss influences the DSB repair decision to favor repair by NHEJ over repair by HR.

To understand how PRMT5 loss affects the DSB repair pathways in MCC, we used irradiation (IR) to induce DSBs in PRMT5i-treated MCC cells. We found that the strongest effect of PRMT5 inhibition was on the ATR/CHK1 pathway, with PRMT5i treatment leading to reduced levels of both ATR and CHK1 phosphorylation in response to IR. ATR is thought to play a role in HR repair after end resection, so it is possible that PRMT5 inhibition interferes with the ability of the cell to repair DSBs by HR. ATR is also a critical regulator of the replication stress response, so we asked whether we saw similar results using a combination of PRMT5i with hydroxyurea

(HU) to induce replication stress. We found that ATR and CHK1 phosphorylation was reduced in both WaGa and MKL-1 lines with the combination treatment compared to HU alone but not in MS-1 cells. The combination of PRMT5i and HU also induced high levels of RPA32 phosphorylation which is associated with higher levels of replication stress suggesting that PRMT5i exacerbates the replication stress induced by HU, possibly by inhibiting the ATR response.

We asked whether this ATR inhibition phenotype was also observed in MS-1 cells, a naturally PRMT5i-resistant line. MS-1 cells in general appeared to have higher levels of ATR and phosphorylated ATR at background compared to the PRMT5i-sensitive MCC lines. ATR phosphorylation also didn't increase much with HU treatment in MS-1 cells in contrast to what we observed in WaGa and MKL-1. We noticed that CHK1 phosphorylation was also less responsive to HU treatment in MS-1 cells compared to MKL-1 and WaGa, although it did seem to decrease with PRMT5i treatment. RPA32 was hyperphosphorylated with the combination treatment in all three cell lines, although the bands were weaker in MS-1 cells compared to WaGa and MKL-1. It's possible that MS-1 cells are less sensitive to replication stress overall compared with MKL-1 and WaGa which could at least partially explain why they are less sensitive to loss of PRMT5. Additional work testing the response of MS-1 cells to replication stress inducers like HU, nucleoside analogs, or topoisomerase inhibitors compared with WaGa or MKL-1 could provide further insight into this theory.

Hyperphosphorylation of RPA32, particularly on S33, is typically associated with ATR activation (reviewed in²¹³). Since we found that the ATR response was defective with PRMT5i, we wanted to know whether this result was specific to PRMT5 inhibition or whether it was a consequence of this defect. We therefore compared the effect of PRMT5 inhibition to ATR inhibition when combined with HU directly and

found in both MKL-1 and WaGa cells that ATR inhibition also led to an increase in RPA32 hyperphosphorylation. These results further indicated that PRMT5 is required for ATR activation.

We wanted to identify the kinase responsible for RPA32 phosphorylation with PRMT5i treatment, so we combined HU treatment in MKL-1 and WaGa cells with an ATM inhibitor (ATMi), an ATR inhibitor (ATRi), or a DNA-PKcs inhibitor (DNAPKi) alone or in combination with PRMT5i. We found that total and phosphorylated ATM levels were reduced with PRMT5i treatment, indicating that PRMT5 activity regulates ATM expression. ATR phosphorylation was also diminished with PRMT5i while DNA-PKcs phosphorylation seemed to increase with PRMT5i treatment. We found that out of all the kinase inhibitors we tested, only ATRi induced high levels of RPA32 phosphorylation when combined with HU. We also saw that RPA32 hyperphosphorylation was unaffected by ATMi but was abolished with DNAPKi suggesting that when ATR is inhibited due to PRMT5i treatment, DNA-PKcs is able to phosphorylate RPA32. We also noticed that CHK2 phosphorylation decreased with ATMi and DNAPKi but increased when ATMi was combined with PRMT5i. CHK2 is canonically thought of as an ATM target but these results suggest that DNA-PKcs activity contributes to CHK2 phosphorylation in response to HU treatment both alone or when combined with PRMT5i in MCC.

From these data, we concluded that PRMT5i inhibits both the ATM and ATR pathways and increases activation of DNA-PKcs leading to DNA-PKcs scavenging of canonical ATM and ATR targets. This conclusion is in alignment with our finding that PRMT5 inhibition increases DSB repair by NHEJ, as DNA-PKcs is a major regulator of this repair pathway. Our proteomic data further support this conclusion: ATM was identified as a downregulated protein while DNA-PKcs was upregulated. Ku80

(*XRCC5*), another protein important for NHEJ repair was also among the significantly upregulated proteins. DNA-PKcs was found to contain at least one DI that increased with PRMT5i treatment, although it's hard to imagine that this would contribute to upregulation of DNA-PKcs expression. More likely, this finding further emphasizes that identifying splicing level changes in a gene is not sufficient to predict the effect on protein expression. In conclusion, we propose a model where PRMT5 inhibition causes a defect in ATR activation which contributes to elevated levels of replication-associated DSBs. Additional work is necessary to identify a mechanism of ATR regulation by PRMT5 and to determine whether rescuing ATR activation could provide resistance to PRMT5 inhibitor.

6.5.1 FUTURE EXPERIMENTS: ADDITIONAL FACTORS INVOLVED IN PRMT5i SENSITIVITY OR RESISTANCE IN MCC.

In this dissertation, we focused on understanding the role of PRMT5 in mRNA splicing regulation in MCC after identifying a number of mRNA splicing and processing factors in our PRMT5i CRISPR screen. In this section, we discuss a few of the other positively and negatively selected genes that may play roles in regulating sensitivity to PRMT5i.

The genes that were positively selected in MKL-1 cells with PRMT5i treatment were enriched for ribonucleoproteins and interestingly, a number of components of the mitochondrial ribosome were included in this ribonucleoprotein GO term category. Proteins such as MRSP30 and MRPL41 and components of the mitochondrial electron transport chain complex I such as NDUFS3 and NDUF5 were among the top positively selected genes. A well described by-product of oxygen metabolism in mitochondria is reactive oxygen species which when dysregulated can cause DNA

damage and cell death (reviewed in²³⁴). PRMT5 has previously been implicated in negatively regulating the mitochondrial biogenesis pathway²³⁵ and a number of studies have shown that PRMT5 loss increases oxidative stress^{169,236}, although the opposite finding was reported by Diao et al.²³⁷. Additional work is necessary to validate these hits and to determine whether PRMT5 inhibition increases levels of reactive oxygen species.

Another interesting finding was that CARM1 (PRMT4) and PRMT1 were both positively selected hits in our screen. Both of these PRMTs are type I PRMT proteins that catalyze the addition of ADMA onto arginine residues. Because both type I and type II PRMTs target glycine- and arginine-rich regions (namely RGG/RG motifs), it is thought that these PRMTs have overlapping targets^{238,239,240}. PRMT1 was previously identified as a negatively selected gene in a targeted CRISPR screen using the PRMT5 inhibitor EPZ-015666 in a small cell lung cancer cell line²⁴⁰. Treatment of both small cell lung cancer and pancreatic cancer lines with a type I PRMT inhibitor MS-023 synergized with EPZ-015666 validating the results of the screen. This finding supports a model where SDMA may compensate for loss of ADMA when type I PRMTs are inhibited and losing both type I and type II PRMT activity leads to unsurmountable defects for the cell. It was therefore surprising to see the top two most highly expressed and well-studied type I PRMTs in our list of positively selected genes.

One possible explanation for this result is that ADMA and SDMA marks have opposing effects in certain contexts. Previous reports have found that E2F1, a member of the E2F family of transcription factors involved in regulating cell cycle progression, is methylated by both PRMT5 and PRMT1 and that these methylation marks have opposing effects on E2F1 expression and activity^{112,158}. Zheng et al. reported that methylation of E2F1 by PRMT1 promotes E2F1 stabilization and E2F1-mediated

apoptosis. In contrast, methylation by PRMT5 reduced expression of E2F1-regulated pro-apoptotic genes and facilitated cell cycle progression¹⁵⁸.

An alternative explanation is that type I PRMTs (PRMT1, CARM1) have reciprocal effects on intron splicing to type II PRMTs (PRMT5). Maron et al. reported that the type I PRMT5 inhibitor MS-023 increased splicing efficiency of retained introns while a PRMT5 inhibitor caused a decrease in intron splicing in A549 cells²⁴¹. It would be interesting to see if type I PRMT inhibitors also increased intron splicing efficiency in MCC and whether loss of PRMT1 or CARM1 could provide resistance to PRMT5i by counteracting the PRMT5i-regulated splicing changes.

6.6 CONCLUSION

The work outlined in this dissertation characterized the activities of TIP60 and PRMT5, two enzymes involved in MCC growth and development. We identify PRMT5, a transcriptional target of ST, L-MYC, and TIP60/P400 complex (SLaP), as a promising therapeutic target in MCC. Our work strengthens the hypothesis that gene transactivation mediated in part by MCV ST activates genes that contribute to MCC growth and that by investigating the activities of this complex we can identify novel therapeutic strategies to treat MCC. By using both targeted and unbiased approaches, we highlighted the importance of arginine methylation in regulating both mRNA splicing and the DNA damage response. Our work has laid the foundation for many future avenues of investigation into the efficacy of PRMT5 inhibitors in MCC treatment as well as further characterization of the role of arginine methylation in MCC biology.



Supplemental Tables

A.1 NEGATIVELY SELECTED CRISPR HITS.

Table A.1: Negatively selected CRISPR hits

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TACC3	10460	0.505405937	-1.896053876	-2.401459813	1
AREG	374	1.204464539	-0.832379837	-2.036844376	2
CD4	920	1.168556014	-0.656155683	-1.824711697	3
FNTB	2342	0.278007121	-1.486057931	-1.764065051	4
SEC61G	23480	0.472992159	-1.250934727	-1.723926885	5
TP53RK	112858	-0.483913243	-2.11901437	-1.635101127	6
PLEKHA7	144100	1.02468165	-0.601919005	-1.626600656	7
HEXIM1	10614	0.157643667	-1.432429389	-1.590073056	8
AXIN2	8313	0.872922264	-0.686945358	-1.559867622	9
FAU	2197	-1.012209772	-2.548988693	-1.536778921	10
PREB	10113	-0.572505078	-2.085927294	-1.513422216	11
FAM69A	388650	0.760762729	-0.701022569	-1.461785297	12
FAM134A	79137	0.750475067	-0.7055498	-1.456024867	13
DEFB106B	503841	0.900334185	-0.548222893	-1.448557078	14
CAPN14	440854	0.800602844	-0.643362314	-1.443965157	15
GLI4	2738	0.821964485	-0.610387855	-1.43235234	16
ZFC3H1	196441	0.65148636	-0.773413217	-1.424899576	17
SETDB1	9869	0.250485988	-1.169467093	-1.419953081	18
CFAP20	29105	0.78585938	-0.610072526	-1.395931906	19
MAP2K4	6416	0.797173623	-0.59730168	-1.394475304	20

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SLC3A2	6520	0.26238997	-1.126109284	-1.388499254	21
KMT2C	58508	0.415263307	-0.956709762	-1.371973069	22
MYCL	4610	-0.239477535	-1.591310419	-1.351832884	23
KLHL12	59349	0.827796344	-0.523109149	-1.350905493	24
PABPN1	8106	-0.041749121	-1.390332898	-1.348583777	25
TBC1D3K	101060351	-0.368193434	-1.716451191	-1.348257757	26
MGST2	4258	0.757682983	-0.587999459	-1.345682442	27
OIP5	11339	0.020672084	-1.312176224	-1.332848308	28
GSPT1	2935	-0.218640107	-1.531082481	-1.312442375	29
DARS2	55157	0.444029443	-0.853259156	-1.297288599	30
CREBZF	58487	0.184802219	-1.108608496	-1.293410715	31
PTCD1	26024	0.178673307	-1.110568044	-1.28924135	32
CLSPN	63967	0.00226547	-1.285373215	-1.287638685	33
MKRN2OS	100129480	0.702029137	-0.585274111	-1.287303249	34
HNRNPH1	3187	-0.597274097	-1.868147214	-1.270873116	35
HOXC5	3222	0.626586287	-0.640817154	-1.267403441	36
NBPF11	200030	0.143378547	-1.123901978	-1.267280524	37
POLR1C	9533	-0.262040495	-1.526082256	-1.264041761	38
COMMD1	150684	0.656335321	-0.602121717	-1.258457038	39
SEC22A	26984	0.59478409	-0.663070409	-1.257854499	40
HSPE1	3336	-1.230544088	-2.482093788	-1.2515497	41

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SRSF7	6432	-0.954459079	-2.202013604	-1.247554526	42
MS4A1	931	0.66679772	-0.573449254	-1.240246974	43
TSPY1	7258	0.207649127	-1.029055363	-1.23670449	44
GMPR2	51292	0.416333574	-0.820149556	-1.23648313	45
ATL1	51062	0.852456152	-0.377269246	-1.229725398	46
ATP6V1B2	526	-0.863551973	-2.09214379	-1.228591817	47
INTS10	55174	0.038383243	-1.178499031	-1.216882274	48
UTP20	27340	-0.355415766	-1.571422136	-1.21600637	49
AQR	9716	0.061736889	-1.148565251	-1.21030214	50
MTA2	9219	0.364043422	-0.843528988	-1.20757241	51
RNF40	9810	-0.063914553	-1.264876796	-1.200962243	52
SMC5	23137	-0.267348142	-1.463556917	-1.196208775	53
LDB1	8861	-0.392416399	-1.582616334	-1.190199935	54
ATXN7L2	127002	0.314723806	-0.875084463	-1.189808269	55
DTL	51514	-0.117436603	-1.306477769	-1.189041166	56
CCP110	9738	0.397287203	-0.790193252	-1.187480455	57
RBM25	58517	-0.491514318	-1.67874679	-1.187232473	58
NRBF2	29982	0.44127733	-0.744920942	-1.186198272	59
GBAS	2631	0.686958041	-0.495360151	-1.182318193	60
C12orf45	121053	0.20223227	-0.977926934	-1.180159203	61
SUMO4	387082	0.453683682	-0.720910852	-1.174594534	62

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
FAM213A	84293	0.569425333	-0.604531736	-1.173957068	63
MS4A10	341116	0.686106197	-0.486846254	-1.172952451	64
STK17A	9263	0.507437258	-0.665345286	-1.172782544	65
CCNJ	54619	0.266299718	-0.904680391	-1.170980109	66
KRBOX4	55634	0.620426796	-0.550204964	-1.17063176	67
GCLC	2729	0.706091781	-0.462205505	-1.168297286	68
CENPN	55839	0.005147544	-1.162079373	-1.167226917	69
UBAP2L	9898	0.215411834	-0.95058336	-1.165995194	70
PCBP2	5094	-0.021235175	-1.185481328	-1.164246153	71
IARS	3376	-0.407290916	-1.563786657	-1.156495741	72
CYP4F12	66002	0.406635651	-0.744583089	-1.15121874	73
KRTAP2-4	85294	0.422318328	-0.728411119	-1.150729517	74
GABRB2	2561	0.411855929	-0.73395198	-1.145807909	75
TMEM14E	645843	0.530589957	-0.614757422	-1.145347378	76
GNB2L1	10399	-0.712600747	-1.856862922	-1.144262175	77
ABCC12	94160	0.624860756	-0.517162935	-1.142023691	78
ACMSD	130013	0.744686893	-0.395963782	-1.140650675	79
TXNDC2	84203	0.546665793	-0.592954638	-1.139620431	80
SPATS1	221409	0.648908984	-0.483174918	-1.132083901	81
UBE2I	7329	-0.473123212	-1.605072301	-1.131949089	82
APOC2	344	0.437891794	-0.693342042	-1.131233836	83

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
ZNF71	58491	0.541379988	-0.58921573	-1.130595719	84
EIF1	10209	0.351877334	-0.775935853	-1.127813187	85
MRPL44	65080	-0.417884368	-1.54495698	-1.127072612	86
USP1	7398	0.020376996	-1.106693995	-1.127070991	87
FRMD1	79981	0.605727017	-0.513356457	-1.119083474	88
EXOC2	55770	-0.228469082	-1.339407181	-1.110938098	89
EXOC7	23265	-0.014634253	-1.124712825	-1.110078572	90
HRH3	11255	0.558285827	-0.5515789	-1.109864727	91
SUPT5H	6829	-0.126243365	-1.232150097	-1.105906731	92
RBMX2	51634	0.549308695	-0.556556602	-1.105865297	93
C4orf45	152940	0.67404932	-0.430379747	-1.104429066	94
CDC23	8697	-0.161642968	-1.264876796	-1.103233828	95
ZNF25	219749	0.438219426	-0.664984909	-1.103204336	96
TEFM	79736	0.192272241	-0.910266228	-1.102538469	97
IKZF5	64376	0.713146801	-0.388643633	-1.101790434	98
KIAA1715	80856	0.735731603	-0.365331772	-1.101063375	99
FAM50A	9130	-0.395998515	-1.494616875	-1.09861836	100
SYT4	6860	0.635454208	-0.462205505	-1.097659713	101
ZBTB40	9923	0.76873512	-0.328888689	-1.097623809	102
CDT1	81620	-0.336238342	-1.432789765	-1.096551424	103
C22orf46	79640	0.506170413	-0.587706653	-1.093877066	104

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
MARS2	92935	-0.519363082	-1.612527591	-1.093164509	105
RPL13	6137	-0.331564118	-1.422789315	-1.091225197	106
PCNP	57092	0.21907695	-0.872088833	-1.091165783	107
MAPK6	5597	0.544437892	-0.546511104	-1.090948996	108
AKAP8L	26993	0.434287836	-0.656245777	-1.090533613	109
RPS26	6231	-0.604241749	-1.690031082	-1.085789333	110
PAIP1	10605	0.608544656	-0.475742151	-1.084286807	111
ARIH1	25820	-0.135633314	-1.218343169	-1.082709855	112
UTP11L	51118	-0.22602276	-1.308482364	-1.082459605	113
FSCB	84075	0.552868969	-0.529167981	-1.082036949	114
ADD3	120	0.51047332	-0.568786882	-1.079260202	115
COPG2	26958	0.563287683	-0.515428623	-1.078716306	116
FOXO3	2309	0.782561213	-0.293954683	-1.076515895	117
FUT10	84750	0.627874975	-0.446866976	-1.074741951	118
ZNF208	7757	0.313304065	-0.760372089	-1.073676154	119
TRAPPC3L	100128327	0.617259682	-0.45619172	-1.073451402	120
SSB	6741	0.382718476	-0.689693229	-1.072411706	121
LRIG2	9860	0.702706245	-0.365624578	-1.068330822	122
MAD2L1	4085	-0.219928795	-1.288188657	-1.068259863	123
LARS	51520	-0.253194417	-1.320712645	-1.067518228	124
MTMR8	55613	0.579407204	-0.487409343	-1.066816546	125

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
LRRIQ3	127255	0.413690671	-0.651065363	-1.064756035	126
GPBAR1	151306	0.431426512	-0.632978963	-1.064405475	127
ICE2	79664	0.550531857	-0.512410469	-1.062942326	128
FBXO11	80204	0.382041369	-0.679782873	-1.061824242	129
OR10J5	127385	0.58641854	-0.474097932	-1.060516472	130
PDZRN3	23024	0.562828998	-0.497409793	-1.060238791	131
PXMP2	5827	0.334032283	-0.725415559	-1.059447842	132
PMP2	5375	0.757770351	-0.301477544	-1.059247895	133
DGAT2	84649	0.580062469	-0.477025992	-1.057088461	134
TLK2	11011	-0.140434222	-1.197261138	-1.056826916	135
ICOSLG	23308	0.525609943	-0.531082481	-1.056692424	136
WTIP	126374	0.447174716	-0.609284202	-1.056458917	137
LSM11	134353	-0.082506607	-1.138294518	-1.055787911	138
THEMIS2	9473	0.351593386	-0.702666787	-1.054260173	139
RPL17	6139	-0.650001092	-1.702486598	-1.052485506	140
GPR37L1	9283	0.347072057	-0.704738952	-1.05181101	141
TMEM170A	124491	0.646528187	-0.405085815	-1.051614002	142
CD200R1L	344807	0.528536793	-0.52178026	-1.050317054	143
GTPBP4	23560	-0.152563178	-1.195143925	-1.042580747	144
ZNF573	126231	0.390385077	-0.65212397	-1.042509047	145
TMEM147	10430	0.235851735	-0.801409973	-1.037261709	146

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
PHYHIP	9796	0.564292423	-0.472791567	-1.03708399	147
PRAMEF12	390999	0.635541577	-0.401504572	-1.037046149	148
AP3M2	10947	0.492213267	-0.544731745	-1.036945012	149
ATP5L	10632	0.193464823	-0.840848687	-1.03431351	150
WDR77	79084	-0.95849988	-1.992454615	-1.033954735	151
OR51A7	119687	0.619007055	-0.414455606	-1.033462661	152
EWSR1	2130	-0.078941965	-1.108473355	-1.029531389	153
METTL23	124512	0.229430138	-0.79553133	-1.024961468	154
BUB3	9184	-1.080816023	-2.103225371	-1.022409347	155
FGFBP3	143282	0.461110019	-0.560836074	-1.021946093	156
SLC43A1	8501	0.450538409	-0.570003153	-1.020541563	157
HRH2	3274	0.669615359	-0.348912113	-1.018527473	158
SMARCD1	6602	0.484131665	-0.53279427	-1.016925935	159
LIN54	132660	-0.012914619	-1.028537322	-1.015622703	160
BTF3	689	0.334447284	-0.681134285	-1.015581569	161
PPP1R10	5514	0.236463316	-0.777760259	-1.014223576	162
IFNA17	3451	0.559945831	-0.452700572	-1.012646404	163
GPR141	353345	0.574317978	-0.437474661	-1.011792639	164
PNMA2	10687	0.338291506	-0.673408712	-1.011700218	165
GPAT2	150763	0.390013761	-0.621401865	-1.011415626	166
DENND6A	201627	0.542057095	-0.463984864	-1.00604196	167

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
BZW2	28969	0.454207894	-0.551804135	-1.006012029	168
SLC25A32	81034	0.5437171	-0.459727916	-1.003445016	169
RPL18	6141	-0.461546862	-1.464773188	-1.003226326	170
RGPD3	653489	0.382893214	-0.6193297	-1.002222913	171
NUP93	9688	-0.099517288	-1.100590117	-1.001072829	172
HTR7	3363	0.562545049	-0.437587279	-1.000132328	173
SF3B5	83443	-1.028460346	-2.028447227	-0.999986882	174
SNX14	57231	0.327523317	-0.67059327	-0.998116586	175
FBXL8	55336	0.45296289	-0.544371368	-0.997334258	176
C5orf47	133491	0.471179259	-0.525181314	-0.996360573	177
SNUPN	10073	0.110329161	-0.885602955	-0.995932117	178
SYT9	143425	0.420199637	-0.57520609	-0.995405728	179
WDFY3	23001	0.538911823	-0.456236767	-0.995148591	180
PLK4	10733	-0.48177271	-1.476890851	-0.995118141	181
UBE2E3	10477	0.48065876	-0.513829452	-0.994488212	182
TRIM43	129868	0.295306118	-0.698319744	-0.993625862	183
RAB3GAP1	22930	0.563746369	-0.429433758	-0.993180127	184
EHMT2	10919	0.187903807	-0.805148881	-0.993052688	185
THOC3	84321	-0.514579647	-1.507185008	-0.992605361	186
PCDHGB7	56099	0.424480702	-0.567345376	-0.991826078	187
AMIGO1	57463	0.448135771	-0.54279472	-0.990930491	188

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TRIM7	81786	0.447633401	-0.542704626	-0.990338027	189
SEC62	7095	0.550269751	-0.438015226	-0.988284977	190
TCHP	84260	0.529781797	-0.458196315	-0.987978112	191
PLA2G10	8399	0.03829369	-0.942700122	-0.980993811	192
LRRC37A2	474170	-0.096533648	-1.075498896	-0.978965248	193
RPL24	6152	-0.818884739	-1.79769359	-0.978808851	194
OR4F17	81099	-0.019979468	-0.997635029	-0.97765556	195
NUDT21	11051	-0.532075224	-1.507635479	-0.975560255	196
ALLC	55821	0.446912609	-0.528312086	-0.975224696	197
RBM6	10180	0.634121836	-0.337222397	-0.971344233	198
SRSF5	6430	0.64323002	-0.326185864	-0.969415884	199
ZCCHC9	84240	-0.336150973	-1.304405604	-0.968254631	200
RPS10-NUDT3	100529239	-0.373872398	-1.341659534	-0.967787136	201
THRAP3	9967	0.357337877	-0.609351773	-0.966689649	202
PSRC1	84722	0.318611712	-0.647912068	-0.96652378	203
HNRNPU	3192	-0.454557368	-1.420672102	-0.966114734	204
CDK5RAP2	55755	0.10458904	-0.860984729	-0.965573769	205
MAGEC2	51438	0.587139331	-0.377314293	-0.964453625	206
NCOA6	23054	0.314286962	-0.648745439	-0.963032401	207
GOLGA8H	728498	-0.274730795	-1.236271904	-0.961541109	208
PCGF1	84759	0.04300723	-0.91639263	-0.95939986	209

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
ERMN	57471	0.390581657	-0.567480517	-0.958062174	210
ZNF281	23528	0.711355743	-0.245844407	-0.957200151	211
TUBB	203068	-0.547932639	-1.504910131	-0.956977492	212
ZNF230	7773	0.455409213	-0.501103653	-0.956512866	213
DEFB136	613210	0.518948081	-0.43256453	-0.951512611	214
OR4M1	441670	0.496953017	-0.454457408	-0.951410425	215
MCOLN2	255231	0.701657821	-0.249380603	-0.951038423	216
LARP1	23367	0.219404582	-0.731001396	-0.950405979	217
FAM175B	23172	0.604918856	-0.344362359	-0.949281215	218
EDC4	23644	0.265906559	-0.681832515	-0.947739074	219
BIVM	54841	0.406045912	-0.539393666	-0.945439579	220
MAGED2	10916	0.666666667	-0.27814316	-0.944809826	221
ELL	8178	-0.432758884	-1.377179152	-0.944420268	222
TAGLN	6876	0.546556582	-0.397653047	-0.94420963	223
DCAF13	25879	-0.471943735	-1.414342988	-0.942399254	224
PPT1	5538	0.553043706	-0.389251768	-0.942295474	225
SCAF4	57466	0.222244064	-0.71771251	-0.939956574	226
CNIH4	29097	0.481095603	-0.458579215	-0.939674818	227
EBLN1	340900	0.413646987	-0.525744403	-0.93939139	228
DRAM2	128338	0.559072145	-0.380152259	-0.939224404	229
SLC9A2	6549	0.615490466	-0.323663228	-0.939153694	230

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
C3orf79	152118	0.635694472	-0.303324474	-0.939018946	231
STRAP	11171	-0.198643601	-1.135411505	-0.936767904	232
HINT3	135114	0.475547692	-0.459908104	-0.935455796	233
C10orf67	256815	0.5491558	-0.386166044	-0.935321844	234
AARS	16	-0.585588537	-1.520654084	-0.935065546	235
PCYOX1L	78991	0.522442828	-0.411459976	-0.933902804	236
DRC1	92749	0.479894284	-0.453804225	-0.933698509	237
SLN	6588	0.537907083	-0.395265553	-0.933172636	238
MTRNR2L2	100462981	0.146609003	-0.786409298	-0.933018301	239
PRAMEF6	440561	-0.081233209	-1.012342898	-0.931109689	240
MTRNR2L7	100288485	0.41246751	-0.518199018	-0.930666528	241
SLC1A3	6507	0.599982526	-0.329857201	-0.929839727	242
CXorf67	340602	0.566520324	-0.358822469	-0.925342794	243
FAM47C	442444	0.648777931	-0.276138565	-0.924916495	244
PHF10	55274	0.312583273	-0.611581603	-0.924164876	245
IFNE	338376	0.666775878	-0.256925988	-0.923701865	246
LRCH1	23143	0.346373108	-0.576917879	-0.923290987	247
CPSF6	11052	-0.159458751	-1.081400063	-0.921941312	248
APC	324	0.603171483	-0.318257579	-0.921429062	249
ARPP21	10777	0.441801542	-0.476643092	-0.918444634	250
MRLN	100507027	0.513684119	-0.40384702	-0.917531139	251

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TECRL	253017	0.527269947	-0.389724762	-0.91699471	252
SLC25A27	9481	0.59528646	-0.320442362	-0.915728822	253
KCNK2	3776	0.397221676	-0.517838641	-0.915060318	254
BCL3	602	0.699320709	-0.215683139	-0.915003847	255
DUX4	100288687	-0.518817028	-1.433352854	-0.914535826	256
IL37	27178	0.465325558	-0.448849047	-0.914174605	257
ZNF778	197320	0.461940021	-0.451574395	-0.913514417	258
ERICH5	203111	0.260686281	-0.652822199	-0.91350848	259
KRTAP5-5	439915	0.443177599	-0.467791342	-0.910968941	260
HOXC9	3225	0.486468777	-0.424433533	-0.91090231	261
FTO	79068	0.362296049	-0.547997658	-0.910293706	262
AUNIP	79000	0.366249481	-0.542704626	-0.908954108	263
ZNF577	84765	0.435467313	-0.473196991	-0.908664304	264
WDR36	134430	-0.01570168	-0.923757827	-0.908056147	265
PPP1R3A	5506	0.520280453	-0.387539979	-0.907820433	266
INCENP	3619	-0.241683594	-1.14888058	-0.907196986	267
UGT2B4	7363	0.462289496	-0.444682193	-0.906971689	268
TSC1	7248	0.214470437	-0.691720348	-0.906190784	269
NDST2	8509	0.252364415	-0.652349205	-0.90471362	270
FSCN3	29999	0.584299849	-0.318077391	-0.90237724	271
FSHR	2492	0.575650351	-0.326636335	-0.902286686	272

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SGCA	6442	0.035580892	-0.864903824	-0.900484717	273
GGPS1	9453	0.123939017	-0.775395288	-0.899334305	274
ZSWIM6	57688	0.526549156	-0.372606874	-0.89915603	275
PAPOLA	10914	-0.389926392	-1.288864363	-0.898937971	276
EXOSC10	5394	-0.128582662	-1.02617235	-0.897589688	277
TRIM52	84851	0.267151563	-0.62964548	-0.896797042	278
OCM	654231	0.515125702	-0.380873012	-0.895998714	279
C15orf62	643338	0.331542276	-0.563921798	-0.895464074	280
SERPINA7	6906	0.555664766	-0.339429704	-0.89509447	281
SFR1	119392	0.322739882	-0.571872607	-0.894612488	282
SOX12	6666	0.491601686	-0.40231542	-0.893917106	283
FST	10468	0.430159666	-0.462025316	-0.892184983	284
LCE1B	353132	0.028193871	-0.863935312	-0.892129183	285
USP29	57663	0.492999585	-0.398396324	-0.891395909	286
CENPA	1058	-0.242666492	-1.133159151	-0.890492659	287
NETO2	81831	0.684271454	-0.205459705	-0.88973116	288
COASY	80347	-0.064519582	-0.952858237	-0.888338655	289
MFSD1	64747	0.439573641	-0.447745394	-0.887319035	290
TINF2	26277	-0.387523753	-1.2744493	-0.886925546	291
RNF8	9025	0.025968154	-0.860939682	-0.886907836	292
CACNA1C	775	0.496865649	-0.389386909	-0.886252558	293

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
CSTA	1475	0.585828801	-0.298526961	-0.884355762	294
SNAPC1	6617	-0.462857392	-1.346569665	-0.883712273	295
NOP14	8602	-0.268527619	-1.152169017	-0.883641397	296
USP22	23326	0.274556058	-0.608856255	-0.883412313	297
IRS4	8471	0.565187952	-0.318054867	-0.883242819	298
ACTN2	88	0.49697486	-0.385783143	-0.882758003	299
EPS8L2	64787	0.503549352	-0.379093653	-0.882643005	300
U2SURP	23350	-0.257104165	-1.139623406	-0.882519241	301
CAAP1	79886	0.520083874	-0.36035407	-0.880437944	302
OXR1	55074	0.502020401	-0.377517005	-0.879537406	303
CD3EAP	10849	-0.527466527	-1.406910221	-0.879443694	304
P4HA3	283208	0.514033593	-0.363687553	-0.877721147	305
AKAP7	9465	0.509752528	-0.367156178	-0.876908706	306
BEND3	57673	0.224406439	-0.651268075	-0.875674514	307
GNLY	10578	0.132468383	-0.743164106	-0.87563249	308
ADSS	159	0.002888408	-0.872494256	-0.875382665	309
DCAF8	50717	0.444466287	-0.429411235	-0.873877521	310
RBMY1F	159163	0.250333093	-0.621829812	-0.872162905	311
NUP50	10762	0.292947164	-0.578021533	-0.870968696	312
UXS1	80146	0.242622808	-0.627145367	-0.869768175	313
BTG1	694	0.423978333	-0.444524528	-0.868502861	314

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
DPP6	1804	0.330188061	-0.538222442	-0.868410504	315
PID1	55022	0.587445122	-0.280553178	-0.8679983	316
PIK3C3	5289	0.201983269	-0.665615568	-0.867598837	317
CCR2	729230	0.456654217	-0.410671652	-0.867325869	318
RABEPK	10244	0.432562305	-0.434479031	-0.867041335	319
SF3A3	10946	-0.131747592	-0.998490923	-0.866743331	320
CDK13	8621	0.060249438	-0.805644398	-0.865893836	321
SDR39U1	56948	0.230565931	-0.635073652	-0.865639583	322
RASGRP4	115727	0.439508114	-0.425987657	-0.865495771	323
RLN2	6019	0.373064238	-0.492161809	-0.865226047	324
FYTTD1	84248	0.658606906	-0.206022794	-0.8646297	325
ACTRT2	140625	0.428849136	-0.435425019	-0.864274155	326
NPAT	4863	0.021301575	-0.842402811	-0.863704386	327
UBN2	254048	0.677631435	-0.185850714	-0.863482149	328
POLR2D	5433	-0.461808968	-1.324924546	-0.863115578	329
FBXL2	25827	0.382019527	-0.478444975	-0.860464502	330
MAP1B	4131	0.504073564	-0.35634488	-0.860418445	331
NFS1	9054	-1.059388856	-1.919208072	-0.859819216	332
EXOSC2	23404	-0.425594653	-1.285395739	-0.859801086	333
CD2BP2	10421	0.275735535	-0.583787558	-0.859523093	334
KRT1	3848	0.383723216	-0.475719627	-0.859442843	335

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
MDN1	23195	-0.274643427	-1.133879905	-0.859236478	336
MRPS36	92259	0.451717887	-0.406977792	-0.858695678	337
CCDC67	159989	0.33289649	-0.524460561	-0.857357051	338
MED1	5469	0.169431885	-0.687260687	-0.856692573	339
TLR1	7096	0.451368412	-0.404522726	-0.855891138	340
PPDPF	79144	0.329729376	-0.52578945	-0.855518826	341
ARPC3	10094	0.067581854	-0.787310239	-0.854892093	342
TRPC5OS	100329135	0.381670052	-0.472115861	-0.853785913	343
COG3	83548	-0.070386388	-0.923915492	-0.853529104	344
VPREB3	29802	0.363759474	-0.488265237	-0.852024711	345
MOAP1	64112	0.40128432	-0.450695977	-0.851980297	346
EIF3I	8668	-0.445514711	-1.29737826	-0.85186355	347
TMEM155	132332	0.433916519	-0.41747376	-0.851390279	348
APOBEC3D	140564	0.314483542	-0.536533177	-0.851016719	349
SSX2B	727837	0.370246598	-0.480652282	-0.85089888	350
IL13RA1	3597	0.310202477	-0.54049732	-0.850699797	351
PROL1	58503	0.506629098	-0.343619082	-0.85024818	352
DTHD1	401124	0.646113186	-0.203232128	-0.849345314	353
C6orf58	352999	0.390035603	-0.458759404	-0.848795006	354
RBM39	9584	-0.519712557	-1.367921978	-0.848209421	355
CNKS3	154043	0.583863006	-0.261746025	-0.845609031	356

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
C15orf43	145645	0.295961383	-0.548921123	-0.844882506	357
KIF4B	285643	0.279579757	-0.56405694	-0.843636696	358
ZBTB3	79842	0.464277133	-0.377967476	-0.842244609	359
SETD3	84193	0.240919118	-0.599283752	-0.84020287	360
NR3C2	4306	0.429133084	-0.41078427	-0.839917354	361
FAM69C	125704	0.58521722	-0.254133069	-0.839350289	362
UCN	7349	0.405958544	-0.433375377	-0.839333921	363
BIRC3	330	0.453028417	-0.385670526	-0.838698942	364
PTGFR	5737	0.57554114	-0.262489301	-0.838030441	365
NUP210	23225	0.44440076	-0.39346367	-0.83786443	366
TES	26136	0.316449337	-0.521239695	-0.837689033	367
CMTR2	55783	0.668850884	-0.168343169	-0.837194052	368
PNLIPRP3	119548	0.554070288	-0.282940673	-0.837010961	369
PCDHB13	56123	0.592490662	-0.243411865	-0.835902528	370
HYDIN	54768	0.31789092	-0.517928736	-0.835819656	371
RMND1	55005	0.46041107	-0.374566422	-0.834977492	372
SLC14A2	8170	0.52628705	-0.306117393	-0.832404442	373
KAZN	23254	0.453399734	-0.37882337	-0.832223104	374
DEF8	54849	0.28857873	-0.543312762	-0.831891492	375
BBS10	79738	0.455562108	-0.374926799	-0.830488907	376
GLMP	112770	0.43966101	-0.390670751	-0.83033176	377

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
DAP	1611	0.371600813	-0.458489121	-0.830089934	378
CBLN1	869	0.522311775	-0.307581423	-0.829893198	379
BECN1	8678	0.366533429	-0.463331682	-0.829865111	380
CCDC106	29903	0.221523273	-0.608203072	-0.829726345	381
ZBTB8B	728116	0.473014001	-0.356682733	-0.829696734	382
SHANK2	22941	0.351964703	-0.47637281	-0.828337513	383
PHLDB1	23187	0.431950724	-0.396256588	-0.828207312	384
SDCCAG3	10807	0.292532163	-0.532929411	-0.825461574	385
LCE2C	353140	0.381757421	-0.442519933	-0.824277354	386
UCMA	221044	0.320774086	-0.500180188	-0.820954275	387
CDK7	1022	-0.371906603	-1.191405018	-0.819498415	388
FFAR4	338557	0.45396763	-0.36551196	-0.81947959	389
THAP4	51078	0.491514318	-0.327514753	-0.81902907	390
SLC4A11	83959	0.415525413	-0.402495608	-0.818021021	391
DNAI2	64446	0.28807636	-0.529595928	-0.817672288	392
KNCN	148930	0.08888452	-0.728681472	-0.817565993	393
CCL16	6360	0.479785073	-0.337019686	-0.816804759	394
UBXN8	7993	0.471987419	-0.344767782	-0.816755201	395
VN1R5	317705	0.27114868	-0.545429974	-0.816578654	396
TFAP2E	339488	0.37952952	-0.436168296	-0.815697816	397
PPP2R5D	5528	0.339274403	-0.475809721	-0.815084124	398

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
OR5K1	26339	0.294410589	-0.519572954	-0.813983543	399
ABCC10	89845	0.374352926	-0.439299068	-0.813651993	400
LCE6A	448835	0.352903916	-0.460651381	-0.813555297	401
ZNF728	388523	0.489177206	-0.323730799	-0.812908004	402
OR1S1	219959	0.501474346	-0.311185189	-0.812659535	403
NENF	29937	0.453421576	-0.358349475	-0.811771051	404
ZFP69B	65243	0.357883931	-0.453691608	-0.811575538	405
GIN52	51659	-0.955660398	-1.766588585	-0.810928187	406
GNG11	2791	0.431775987	-0.378192711	-0.809968698	407
MANEA	79694	0.482580871	-0.326501194	-0.809082064	408
CRNKL1	51340	-0.062804971	-0.871503221	-0.80869825	409
MYEOV2	150678	0.149358932	-0.658835984	-0.808194916	410
ABLIM2	84448	0.392896927	-0.414996171	-0.807893098	411
INHBA	3624	0.484546666	-0.322987522	-0.807534188	412
CTAGE5	4253	0.508900684	-0.298572008	-0.807472691	413
WDR25	79446	-0.260620754	-1.066939952	-0.806319198	414
PYGO2	90780	0.343730206	-0.462182981	-0.805913187	415
ATXN2	6311	0.324880414	-0.480787423	-0.805667837	416
SERBP1	26135	0.16052028	-0.644578585	-0.805098865	417
BOP1	23246	-0.487517201	-1.291657282	-0.804140081	418
CLTA	1211	0.428805452	-0.375152034	-0.803957486	419

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
PABPC1	26986	-0.337810978	-1.141582954	-0.803771976	420
TMEM131	23505	0.164908372	-0.638632371	-0.803540743	421
RPS13	6207	-0.749666907	-1.55304293	-0.803376023	422
LRRC16B	90668	0.278553175	-0.524798414	-0.803351589	423
TIMM23	100287932	0.169728939	-0.633429434	-0.803158372	424
SHCBP1	79801	0.371557128	-0.431528447	-0.803085575	425
OR52H1	390067	0.543542363	-0.2590432	-0.802585563	426
DEFB130	245940	0.13447131	-0.667890446	-0.802361756	427
SERPINA12	145264	0.413035406	-0.388463444	-0.80149885	428
NMRK2	27231	0.229342769	-0.572097842	-0.801440611	429
SUGCT	79783	0.671974314	-0.129404478	-0.801378791	430
FCGR1B	2210	0.143492126	-0.657777377	-0.801269503	431
C19orf43	79002	-0.167791538	-0.968309383	-0.800517845	432
FBL	2091	-0.571565865	-1.372066309	-0.800500444	433
CEP112	201134	0.458488959	-0.341749628	-0.800238587	434
CYP1B1	1545	0.323307778	-0.476845804	-0.800153582	435
MAPK11	5600	0.342900203	-0.457182756	-0.800082959	436
SLC20A2	6575	0.428652557	-0.371142844	-0.799795401	437
TCEAL8	90843	0.56466374	-0.234695257	-0.799358996	438
SLC25A51	92014	-0.058207195	-0.856637686	-0.798430492	439
ZDHHC5	25921	0.475897167	-0.322514528	-0.798411695	440

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
DEFA4	1669	0.472315051	-0.324902023	-0.797217074	441
FAM200A	221786	0.343642837	-0.453286184	-0.796929021	442
ADHFE1	137872	0.463316078	-0.332942925	-0.796259003	443
VEZF1	7716	0.29764323	-0.498017929	-0.795661159	444
CSTL1	128817	0.526461787	-0.268795892	-0.795257679	445
ANGPTL1	9068	0.435292576	-0.35889004	-0.794182616	446
RNF166	115992	0.350370225	-0.44357854	-0.793948764	447
ARHGAP29	9411	0.437454951	-0.356119645	-0.793574596	448
CCDC82	79780	0.323220409	-0.469863507	-0.793083917	449
AKIRIN2	55122	-0.290347946	-1.082819046	-0.7924711	450
DAZAP2	9802	0.072926632	-0.719514393	-0.792441025	451
PRKACB	5567	0.302382981	-0.489909455	-0.792292436	452
UBE2E2	7325	0.426730446	-0.365489436	-0.792219882	453
EIF3J	8669	0.103809274	-0.688116582	-0.791925856	454
RYR3	6263	0.461284756	-0.330510383	-0.79179514	455
ADAM22	53616	0.435947841	-0.355398892	-0.791346733	456
BTBD11	121551	0.315750388	-0.475314203	-0.791064591	457
SPPL2A	84888	0.376275037	-0.413847471	-0.790122507	458
HEY2	23493	0.362274207	-0.427834587	-0.790108794	459
ERVV-2	100271846	0.455234476	-0.333979008	-0.789213484	460
FXR1	8087	0.50110303	-0.28706248	-0.78816551	461

Table A.1: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
ZNF157	7712	0.494441168	-0.293346547	-0.787787715	462
CFHR2	3080	0.348972326	-0.438578314	-0.78755064	463
IP6K2	51447	0.279885547	-0.507612956	-0.787498503	464
RRAGD	58528	0.397789573	-0.389206721	-0.786996294	465
DROSHA	29102	-0.146707293	-0.933510518	-0.786803225	466
FIBP	9158	0.591311185	-0.195490788	-0.786801973	467
SPINK14	408187	0.412795142	-0.37366548	-0.786460623	468

A.2 POSITIVELY SELECTED CRISPR HITS.

Table A.2: Positively selected CRISPR hits

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
ATP5E	514	-2.300198764	-0.331276184	1.96892258	18967
NDUFB5	4711	-1.545093157	0.305666922	1.850760079	18966
DAD1	1603	-2.553131075	-0.78145412	1.771676955	18965
CARM1	10498	-0.88104755	0.848979684	1.730027234	18964
MRPL41	64975	-2.131118537	-0.440132438	1.690986099	18963
WARS2	10352	-1.404538803	0.2590432	1.663582003	18962
MRPS30	10884	-1.70991416	-0.046988603	1.662925557	18961
C14orf169	79697	-0.652447415	0.979954052	1.632401467	18960
GPN2	54707	-2.818076579	-1.215460156	1.602616423	18959
SLC35F3	148641	-0.561955311	1.016059282	1.578014593	18958
KRR1	11103	-1.429788349	0.100984279	1.530772628	18957
PLK1	5347	-2.223095909	-0.731541961	1.491553948	18956
DTYMK	1841	-1.64222528	-0.183017253	1.459208027	18955
CDC20	991	-1.814887622	-0.355849363	1.459038259	18954
WDR72	256764	-0.71242601	0.70230641	1.41473242	18953
ORC6	23594	-1.793984667	-0.391909545	1.402075121	18952
POP5	51367	-1.64816635	-0.251948286	1.396218064	18951
NDUFS3	4722	-1.329489112	0.06669895	1.396188062	18950
PYROXD2	84795	-0.862831182	0.528267039	1.391098221	18949

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
EIF5	1983	-1.795688356	-0.413667282	1.382021074	18948
SLC8A3	6547	-0.628508398	0.752556421	1.38106482	18947
SESN1	27244	-0.575803246	0.803437092	1.379240338	18946
OR4F21	441308	-1.381167682	-0.014523627	1.366644055	18945
ATP5SL	55101	-1.310202477	0.055563314	1.365765791	18944
TOMM20	9804	-1.187689754	0.163617731	1.351307484	18943
EXOSC5	56915	-1.189284232	0.160090545	1.349374777	18942
STX1A	6804	-0.545792106	0.798481914	1.34427402	18941
CCDC81	60494	-0.451171832	0.893080769	1.344252602	18940
ZRANB2	9406	-0.798396785	0.531487905	1.32988469	18939
CXADR	1525	-0.573881135	0.747466102	1.321347237	18938
BMP8A	353500	-0.927025315	0.391504122	1.318529437	18937
WWC3	55841	-0.500338554	0.811996036	1.312334589	18936
CD14	929	-0.67385274	0.638339565	1.312192305	18935
EDDM3B	64184	-0.494681432	0.809405829	1.304087261	18934
ZNF786	136051	-0.486141144	0.816298031	1.302439176	18933
THAP11	57215	-1.164296791	0.136551196	1.300847987	18932
CELF4	56853	-0.50372409	0.793504212	1.297228302	18931
PAXBP1	94104	-1.344145207	-0.053013649	1.291131557	18930
ATP8B2	57198	-0.604067012	0.68313888	1.287205892	18929
RAE1	8480	-1.514667016	-0.231384297	1.28328272	18928

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
DHX57	90957	-0.677544067	0.602437047	1.279981113	18927
FAM127B	26071	-0.31557565	0.957858462	1.273434112	18926
ZNF709	163051	-0.594915143	0.675323213	1.270238356	18925
RUNDC3A	10900	-0.637157897	0.619487364	1.256645261	18924
NUS1	116150	-1.852761942	-0.596941304	1.255820639	18923
HSFX1	100506164	-0.581241946	0.668003063	1.249245009	18922
GCSH	2653	-1.120699823	0.127776026	1.248475849	18921
SLC25A30	253512	-0.61573073	0.625433578	1.241164308	18920
YPEL1	29799	-1.000677107	0.234988063	1.23566517	18919
NEURL1	9148	-0.610379398	0.623609172	1.23398857	18918
XKRY	9082	-0.480746128	0.752488851	1.233234979	18917
EDN1	1906	-0.821156324	0.411955493	1.233111818	18916
MDC1	9656	-0.978594675	0.253795216	1.232389891	18915
MAFG	4097	-0.888473888	0.342740664	1.231214552	18914
ATP5I	521	-1.528820741	-0.302130727	1.226690015	18913
NMS	129521	-0.724242623	0.499932429	1.224175052	18912
ATP6V0B	533	-1.571150864	-0.350286049	1.220864815	18911
EIF6	3692	-1.782823319	-0.56412451	1.218698809	18910
SRM	6723	-1.022912435	0.194902924	1.217815358	18909
RTF1	23168	-1.245637027	-0.031041939	1.214595088	18908
GPR61	83873	-0.702247559	0.51177981	1.214027369	18907

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TRIM43B	653192	-0.820675797	0.391661787	1.212337583	18906
MRPS35	60488	-1.55791451	-0.357178251	1.200736258	18905
VPS54	51542	-0.815804993	0.383508266	1.199313259	18904
TALDO1	6888	-0.713889435	0.484458759	1.198348194	18903
TSPY10	100289087	-1.567131905	-0.377359341	1.189772564	18902
TIMM13	26517	-0.941965358	0.24726339	1.189228749	18901
KIAA0907	22889	-0.619465741	0.56820127	1.187667011	18900
DDX51	317781	-1.179083939	0.00717735	1.18626129	18899
XRN2	22803	-0.861149335	0.324496599	1.185645934	18898
GTF3C3	9330	-1.349147063	-0.166243975	1.182903088	18897
NACC2	138151	-0.485944565	0.689940988	1.175885553	18896
CD300E	342510	-0.746914794	0.428442723	1.175357516	18895
MRPL17	63875	-1.457702641	-0.28298572	1.174716921	18894
FABP9	646480	-0.658934539	0.513153746	1.172088285	18893
CXXC1	30827	-0.885787301	0.284224515	1.170011816	18892
NDUFA3	4696	-1.085752353	0.076485427	1.162237781	18891
HTR3A	3359	-0.509250158	0.645547097	1.154797255	18890
POLR2J3	548644	-1.412511194	-0.258299923	1.154211271	18889
KANSL1L	151050	-0.646615556	0.506757061	1.153372617	18888
SIX4	51804	-0.667867986	0.482228929	1.150096915	18887
XRCC5	7520	-1.748705852	-0.600680211	1.148025641	18886

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
NAA20	51126	-1.532664963	-0.385197531	1.147467432	18885
MCRS1	10445	-0.975667824	0.170777963	1.146445787	18884
C6orf1	221491	-0.612017561	0.533132123	1.145149684	18883
GPS1	2873	-1.301159819	-0.156504798	1.144655022	18882
SLC17A1	6568	-0.513487539	0.626830037	1.140317576	18881
FAM111B	374393	-0.500120132	0.639443218	1.13956335	18880
CCDC84	338657	-1.467902933	-0.329429254	1.13847368	18879
C15orf41	84529	-1.284341349	-0.148923375	1.135417974	18878
SCX	642658	-0.58934539	0.545948016	1.135293406	18877
KCNJ14	3770	-0.705698622	0.426077751	1.131776373	18876
GYPB	2994	-0.43915864	0.686742646	1.125901286	18875
ATP6V0C	527	-1.603783064	-0.481327988	1.122455076	18874
PDGFA	5154	-0.43422231	0.687778729	1.122001038	18873
MEGF10	84466	-0.609309132	0.510135592	1.119444724	18872
BACE2	25825	-0.464211607	0.653497905	1.117709512	18871
TUBB4B	10383	-0.965642269	0.148610298	1.114252567	18870
TMEM121	80757	-0.52266125	0.589305825	1.111967074	18869
SAV1	60485	-0.730948169	0.379071129	1.110019298	18868
COPRS	55352	-0.734049756	0.375467363	1.10951712	18867
QRSL1	55278	-1.350959963	-0.241970359	1.108989604	18866
BUD31	8896	-1.862678287	-0.754380828	1.108297459	18865

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
GPRC6A	222545	-0.580302732	0.527613856	1.107916589	18864
TMPRSS11A	339967	-0.529650744	0.57660255	1.106253293	18863
ELMOD2	255520	-0.609003342	0.495743051	1.104746393	18862
SKP1	6500	-1.335124391	-0.233524033	1.101600359	18861
C10orf142	100130539	-0.553305812	0.547975134	1.101280946	18860
HNRNPCL4	101060301	-0.650175829	0.447227353	1.097403182	18859
C7orf77	154872	-0.62116943	0.476192621	1.097362051	18858
TMEM106C	79022	-0.556363716	0.539663949	1.096027665	18857
CLDN23	137075	-0.443330494	0.651448263	1.094778757	18856
USP17L19	100287404	-1.321145403	-0.22802829	1.093117114	18855
GPM6A	2823	-0.468208724	0.623901978	1.092110701	18854
SSU72	29101	-1.327479632	-0.236069192	1.09141044	18853
ZFYVE28	57732	-0.545945001	0.545362404	1.091307405	18852
ZHX3	23051	-0.386606382	0.702081175	1.088687557	18851
SRP19	6728	-1.260992071	-0.172602369	1.088389702	18850
AMTN	401138	-0.448376035	0.639420695	1.087796729	18849
ESRP2	80004	-0.815149728	0.270980675	1.086130403	18848
SGK494	124923	-0.899045497	0.185357449	1.084402946	18847
ARNT2	9915	-0.569665596	0.514234875	1.083900472	18846
NPIPA1	9284	-1.456545006	-0.374025857	1.082519149	18845
RPL5	6125	-1.747067689	-0.670232893	1.076834796	18844

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
COLQ	8292	-0.51440491	0.561399162	1.075804072	18843
MPV17L2	84769	-0.651442675	0.420942385	1.07238506	18842
PRMT1	3276	-1.493108796	-0.421460426	1.07164837	18841
PHB2	11331	-1.564270581	-0.493040227	1.071230354	18840
NUMB	8650	-0.623200751	0.443938916	1.067139668	18839
FAM195B	348262	-0.67898565	0.386413802	1.065399452	18838
COQ10A	93058	-0.633990783	0.428983288	1.06297407	18837
RPS17	6218	-1.580586681	-0.51840173	1.062184951	18836
DLEC1	9940	-0.743288994	0.31686112	1.060150114	18835
CYP4A11	1579	-0.734617653	0.325284923	1.059902576	18834
SPATA31A5	727905	-0.820697639	0.236519663	1.057217302	18833
ANKRD18A	253650	-0.569621912	0.486215595	1.055837507	18832
VEGFB	7423	-0.61935653	0.43333033	1.05268686	18831
DDTL	100037417	-0.892296267	0.159905852	1.052202119	18830
FRG2B	441581	-0.804250486	0.247849002	1.052099488	18829
C11orf49	79096	-0.695782277	0.351592414	1.047374691	18828
LRRC56	115399	-0.578227726	0.468039101	1.046266827	18827
LRIT2	340745	-0.313194854	0.732983468	1.046178322	18826
IDO1	3620	-0.277679488	0.766385873	1.044065361	18825
VDR	7421	-0.444116812	0.599441416	1.043558228	18824
DEAF1	10522	-0.830461088	0.212885716	1.043346804	18823

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
FGFR1OP	11116	-0.740383985	0.301950538	1.042334524	18822
CYP27B1	1594	-0.607539917	0.434366413	1.041906329	18821
H2AFV	94239	-0.625363126	0.414410559	1.039773685	18820
TADA3	10474	-0.997357098	0.04241182	1.039768918	18819
KIRREL2	84063	-0.652884258	0.38652642	1.039410678	18818
SASH3	54440	-0.366970273	0.670503176	1.037473449	18817
PSMD1	5707	-1.080313654	-0.049799541	1.030514113	18816
MTRF1L	54516	-1.195290828	-0.167268796	1.028022033	18815
TMSB15B	286527	-0.354411026	0.671651876	1.026062902	18814
ADAMTS3	9508	-0.361029203	0.664894815	1.025924018	18813
UGT2B7	7364	-0.46021449	0.563133474	1.023347965	18812
ALDH1B1	219	-0.644780814	0.374904275	1.019685089	18811
NCAPH2	29781	-0.824061333	0.195535835	1.019597168	18810
SRSF3	6428	-2.145381473	-1.125793955	1.019587519	18809
AKAP1	8165	-0.612432562	0.405941709	1.018374271	18808
TRPV1	7442	-0.59891226	0.419163025	1.018075285	18807
HIST1H1B	3009	-0.540331564	0.477611604	1.017943168	18806
GPM6B	2824	-0.302448507	0.715370062	1.017818569	18805
RPF2	84154	-1.536836817	-0.522501014	1.014335804	18804
MBD6	114785	-0.859576699	0.153319969	1.012896668	18803
USH2A	7399	-0.469104253	0.543650615	1.012754868	18802

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
FBF1	85302	-0.628814189	0.383710978	1.012525167	18801
SYMPK	8189	-1.327130157	-0.315239425	1.011890732	18800
LHB	3972	-0.711268375	0.298932384	1.010200759	18799
KIF17	57576	-0.54985475	0.459299968	1.009154718	18798
JUP	3728	-0.491143001	0.517568359	1.00871136	18797
ANKRD36	375248	-0.524212044	0.483400153	1.007612197	18796
DRD4	1815	-0.407771444	0.596986351	1.004757794	18795
OR52E6	390078	-0.471878208	0.531938376	1.003816584	18794
SQRDL	58472	-0.655068475	0.34596153	1.001030005	18793
FAM171A2	284069	-0.753773235	0.245303843	0.999077077	18792
RPS4X	6191	-2.23358015	-1.234988063	0.998592087	18791
DCAF7	10238	-1.319332503	-0.32163611	0.997696394	18790
TNRC6B	23112	-0.690037787	0.307040858	0.997078645	18789
GTF3C4	9329	-0.670379835	0.325420064	0.995799899	18788
DPCR1	135656	-0.438940218	0.556849408	0.995789626	18787
EXOSC1	51013	-1.003844222	-0.008130997	0.995713225	18786
NPAS4	266743	-0.576218247	0.419410784	0.995629031	18785
MAPK8IP1	9479	-0.763230894	0.230348214	0.993579107	18784
TMEM109	79073	-0.623331804	0.369813956	0.99314576	18783
KCNE5	23630	-0.436734159	0.553313212	0.990047371	18782
OPN4	94233	-0.381473473	0.606784089	0.988257562	18781

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SLC10A6	345274	-0.28021318	0.707531871	0.98774505	18780
HSD17B10	3028	-1.201100845	-0.213953331	0.987147514	18779
ANO6	196527	-0.551951598	0.435019595	0.986971193	18778
GPR174	84636	-0.230871721	0.755259246	0.986130967	18777
RPS6	6194	-2.399799052	-1.414658318	0.985140734	18776
SMARCAD1	56916	-0.825786864	0.158365242	0.984152106	18775
GTF3C1	2975	-0.685166983	0.297513402	0.982680385	18774
TESC	54997	-0.37983531	0.600342358	0.980177668	18773
TRIM21	6737	-0.420199637	0.559372044	0.979571681	18772
SCUBE3	222663	-0.651573728	0.325983152	0.977556881	18771
KLHL21	9903	-0.395081144	0.58067931	0.975760454	18770
DBN1	1627	-0.38627875	0.589395919	0.975674668	18769
KIR3DL1	3811	-0.221654326	0.752218568	0.973872894	18768
BRWD3	254065	-0.426359129	0.545362404	0.971721533	18767
C5orf67	101928448	-0.612585457	0.35850714	0.971092597	18766
PTPN21	11099	-0.467029247	0.503356007	0.970385254	18765
GATSL2	729438	-0.385710853	0.584643452	0.970354306	18764
CNTN6	27255	-0.608391761	0.360894635	0.969286396	18763
PITPNB	23760	-0.688421466	0.280598225	0.969019692	18762
TLCD1	116238	-0.616517048	0.351209514	0.967726562	18761
SUCLA2	8803	-0.625887338	0.341817199	0.967704537	18760

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
NDUFB9	4715	-1.08551209	-0.117813865	0.967698224	18759
RPL4	6124	-1.431077037	-0.463579441	0.967497597	18758
USP39	10713	-1.371513444	-0.405288527	0.966224917	18757
POLR2H	5437	-0.926020575	0.04011442	0.966134995	18756
IL2RG	3561	-0.397745888	0.567457994	0.965203882	18755
SCP2D1	140856	-0.453508944	0.511261769	0.964770713	18754
OSTC	58505	-0.658934539	0.305824587	0.964759126	18753
ARL5A	26225	-0.427538606	0.537141313	0.964679919	18752
ATP8A1	10396	-0.438437848	0.526014685	0.964452533	18751
SYT12	91683	-0.76823275	0.195920987	0.964153738	18750
NOC3L	64318	-0.664504292	0.299472949	0.963977241	18749
LYRM9	201229	-0.807679706	0.154079013	0.961758719	18748
FNTA	2339	-1.250660726	-0.289427452	0.961233274	18747
KIAA1033	23325	-0.499180919	0.460786522	0.959967441	18746
MESDC1	59274	-0.605202805	0.354610568	0.959813373	18745
PTCD3	55037	-1.228469082	-0.268705798	0.959763285	18744
CTAGE6	340307	-0.558001878	0.401459525	0.959461404	18743
MYOZ3	91977	-0.487276937	0.471620343	0.95889728	18742
PRDX3	10935	-0.497346177	0.457948556	0.955294733	18741
CAPNS2	84290	-0.384138217	0.571039236	0.955177453	18740
PSKH1	5681	-0.491973003	0.462948781	0.954921785	18739

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
USP17L25	728373	-1.592556189	-0.638474706	0.954081483	18738
ATP1A1	476	-0.324378044	0.629555385	0.95393343	18737
KIR2DL1	3802	-0.540855776	0.412563629	0.953419405	18736
ZNF599	148103	-0.631522618	0.320600027	0.952122645	18735
KIF22	3835	-0.659720857	0.291386999	0.951107857	18734
MEF2B	100271849	-0.706921783	0.243637101	0.950558884	18733
C10orf105	414152	-0.607452548	0.341321681	0.948774229	18732
FRG2C	100288801	-0.627154184	0.321455921	0.948610105	18731
SLC16A8	23539	-0.704125986	0.243659624	0.94778561	18730
HYPM	25763	-0.393901667	0.549979729	0.943881395	18729
C9orf114	51490	-1.086604198	-0.14272715	0.943877048	18728
NCAPH	23397	-1.292663216	-0.350578855	0.942084361	18727
TFCP2	7024	-0.45910054	0.482927159	0.942027698	18726
PFN2	5217	-0.428805452	0.512770846	0.941576297	18725
RASGRP3	25780	-0.392940611	0.548493175	0.941433787	18724
ALS2	57679	-0.571369286	0.368755349	0.940124635	18723
ST8SIA4	7903	-0.332940174	0.606401189	0.939341364	18722
C9orf172	389813	-0.472467947	0.466304789	0.938772735	18721
ORC1	4998	-1.327392264	-0.388666156	0.938726107	18720
TAF6L	10629	-1.068933884	-0.130557683	0.938376201	18719
EMILIN3	90187	-0.361378678	0.576940403	0.93831908	18718

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
STAMBP	10617	-0.601751742	0.335533132	0.937284874	18717
MEGF9	1955	-0.464058712	0.472836614	0.936895326	18716
PDE12	201626	-1.677762488	-0.741159512	0.936602977	18715
IFNA16	3449	-0.411375401	0.525046173	0.936421575	18714
ABI3BP	25890	-0.557848983	0.377314293	0.935163277	18713
GMCL1	64395	-0.540331564	0.393621334	0.933952898	18712
TUBA4A	7277	-0.54290894	0.39022028	0.93312922	18711
AGFG2	3268	-0.595417513	0.33672688	0.932144393	18710
SNTG2	54221	-0.425900443	0.506216496	0.93211694	18709
RASL11B	65997	-0.453093943	0.478940493	0.932034436	18708
HSP90AB1	3326	-0.85112378	0.080436056	0.931559835	18707
HMGB2	3148	-0.59659699	0.334542096	0.931139087	18706
ZNF787	126208	-0.421357272	0.509775215	0.931132487	18705
C3orf33	285315	-0.441517594	0.48936889	0.930886484	18704
DRAP1	10589	-0.819976847	0.109998198	0.929975045	18703
NDUFS5	4725	-1.4218378	-0.49229695	0.92954085	18702
NUF2	83540	-0.862394339	0.06692869	0.929323029	18701
SF3B3	23450	-1.858571959	-0.929298617	0.929273342	18700
UPK1A	11045	-0.588471703	0.33972251	0.928194213	18699
GTF3C5	9328	-1.051613044	-0.125654309	0.925958735	18698
BCAS1	8537	-0.46978136	0.455718726	0.925500086	18697

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
B4GALT7	11285	-0.256164952	0.669241858	0.92540681	18696
POLR1B	84172	-1.211869034	-0.286499392	0.925369642	18695
OR6P1	128366	-0.455147107	0.469773413	0.92492052	18694
CASP9	842	-0.528514951	0.396279112	0.924794063	18693
C6orf25	80739	-0.291920582	0.632190639	0.924111221	18692
RPS28	6234	-1.808684446	-0.885355196	0.92332925	18691
ASF1A	25842	-0.458336064	0.46173251	0.920068574	18690
CDC73	79577	-1.299041129	-0.379026082	0.920015047	18689
KATNAL1	84056	-0.696240963	0.222705978	0.918946941	18688
OR51T1	401665	-0.509337527	0.409275193	0.91861272	18687
OR2H2	7932	-0.347661796	0.570859048	0.918520844	18686
GMEB2	26205	-0.553393181	0.36404793	0.917441111	18685
AWAT2	158835	-0.272655789	0.643767737	0.916423527	18684
INTS3	65123	-1.219775899	-0.303482139	0.916293761	18683
CYP17A1	1586	-0.548325798	0.367944502	0.9162703	18682
ZFAND4	93550	-0.446257344	0.468985089	0.915242434	18681
SPATA5	166378	-1.301902453	-0.387720168	0.914182285	18680
KCNA5	3741	-0.380621628	0.53241137	0.913032998	18679
FZD9	8326	-0.656313479	0.254943916	0.911257395	18678
SHMT2	6472	-0.762903261	0.146513356	0.909416617	18677
ZBTB24	9841	-0.50121224	0.408171539	0.90938378	18676

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
MED9	55090	-0.77236092	0.136521915	0.908882835	18675
NMUR1	10316	-0.522005985	0.38684175	0.908847734	18674
TAF1C	9013	-1.293930061	-0.385895761	0.9080343	18673
FTSJ2	29960	-1.069086779	-0.161651426	0.907435353	18672
SGCB	6443	-0.466439508	0.440380197	0.906819705	18671
ARMC12	221481	-0.585959854	0.320757692	0.906717546	18670
ARPC1A	10552	-0.687307516	0.219181044	0.90648856	18669
H2AFB3	83740	-0.836555053	0.068696788	0.905251841	18668
MT1A	4489	-0.766965904	0.137713411	0.904679315	18667
UXT	8409	-1.02961798	-0.124987612	0.904630368	18666
ANKRD52	283373	-0.591595134	0.313032119	0.904627252	18665
MAEL	84944	-0.305113252	0.599103563	0.904216815	18664
CDH15	1013	-0.60153332	0.301432497	0.902965817	18663
LDOC1L	84247	-0.356486032	0.546398486	0.902884518	18662
CCDC79	283847	-0.372561868	0.530046398	0.902608266	18661
KIAA0754	643314	-0.615577835	0.286724627	0.902302462	18660
ABHD5	51099	-0.501518031	0.400738772	0.902256803	18659
LRP8	7804	-0.840726907	0.061324834	0.902051742	18658
TNFRSF19	55504	-0.480920866	0.421009955	0.901930821	18657
DTX3L	151636	-0.39746194	0.502319924	0.899781864	18656
SPAG16	79582	-0.527182579	0.372314068	0.899496647	18655

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
DDX49	54555	-1.054823843	-0.157284112	0.897539731	18654
ZMYM6NB	100506144	-0.329445427	0.567795847	0.897241274	18653
FAM86C1	55199	-0.66548719	0.230776161	0.896263351	18652
APBB3	10307	-0.612301509	0.283503761	0.895805271	18651
SSNA1	8636	-0.362645523	0.532816794	0.895462317	18650
SOD2	6648	-1.456872638	-0.562908239	0.893964399	18649
SLC4A4	8671	-0.481226656	0.412653723	0.893880379	18648
IL6ST	3572	-0.525675469	0.368034596	0.893710065	18647
TRIT1	54802	-0.738396348	0.154923645	0.893319993	18646
GPC4	2239	-0.281611078	0.610815803	0.892426881	18645
MYBL2	4605	-1.434724679	-0.543695662	0.891029017	18644
SMIM1	388588	-0.388069808	0.50216226	0.890232067	18643
ANKRD39	51239	-0.095380381	0.793977206	0.889357588	18642
RRP9	9136	-1.108468209	-0.219388261	0.889079948	18641
LSM2	57819	-1.721971037	-0.833641155	0.888329882	18640
RPL15	6138	-1.501386978	-0.61315825	0.888228727	18639
SCAP	22937	-0.941047987	-0.053838011	0.887209977	18638
C10orf35	219738	-0.432868095	0.453736655	0.88660475	18637
SYCE3	644186	-0.460236332	0.426212892	0.886449225	18636
DDX52	11056	-0.982176791	-0.095803865	0.886372925	18635
ANXA1	301	-0.444641024	0.441281139	0.885922163	18634

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
RPS6KA3	6197	-0.403774327	0.481800982	0.885575309	18633
APEX1	328	-0.522573881	0.36289923	0.885473111	18632
RPAIN	84268	-0.680711181	0.204542997	0.885254178	18631
CMTM5	116173	-0.470873468	0.413284382	0.88415785	18630
FSCN2	25794	-0.636502632	0.247398531	0.883901163	18629
AP2M1	1173	-0.889063626	-0.006847155	0.882216471	18628
MFSD6	54842	-0.531922329	0.349790531	0.88171286	18627
VSTM2L	128434	-0.548609746	0.332064507	0.880674253	18626
LPPR3	79948	-0.494856169	0.385332673	0.880188842	18625
DOT1L	84444	-1.005875543	-0.125965134	0.87991041	18624
TMPRSS7	344805	-0.446497608	0.433397901	0.879895509	18623
NDUFS2	4720	-1.044885656	-0.166066039	0.878819617	18622
C16orf74	404550	-0.444946814	0.433825848	0.878772662	18621
GRAPL	400581	-0.630867353	0.247443579	0.878310931	18620
RFC2	5982	-1.147543848	-0.269268886	0.878274962	18619
HIGD1B	51751	-0.55902846	0.317852156	0.876880616	18618
H3F3A	3020	-1.7290479	-0.852178026	0.876869874	18617
C3orf38	285237	-0.582421423	0.294270012	0.876691435	18616
OR2A7	401427	-0.552519494	0.324068652	0.876588146	18615
C19orf53	28974	-1.334840443	-0.459345016	0.875495427	18614
FNDC3B	64778	-0.394382194	0.481102752	0.875484947	18613

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
NDUFB10	4716	-1.279470546	-0.404477679	0.874992867	18612
AGO3	192669	-0.624314702	0.249650885	0.873965587	18611
NCR2	9436	-0.531682065	0.34226767	0.873949735	18610
PDCD5	9141	-0.463512658	0.410153611	0.873666268	18609
ZCCHC16	340595	-0.266736562	0.606784089	0.873520651	18608
SGSM1	129049	-0.611886508	0.261610883	0.873497391	18607
CTTNBP2	83992	-0.403293799	0.468151719	0.871445518	18606
CD48	962	-0.438612585	0.432519483	0.871132068	18605
CEP85	64793	-0.751501649	0.119509888	0.871011537	18604
RNF10	9921	-0.526876788	0.343641605	0.870518394	18603
NAMPT	10135	-0.683943822	0.185846209	0.869790031	18602
CXCL16	58191	-0.488784046	0.380805442	0.869589488	18601
CD163	9332	-0.412838827	0.45650705	0.869345877	18600
GOLM1	51280	-0.408164603	0.461011757	0.86917636	18599
ACPT	93650	-0.214072909	0.653678094	0.867751003	18598
FAM102A	399665	-0.548871852	0.318595432	0.867467284	18597
FCER2	2208	-0.472642684	0.394251993	0.866894677	18596
ST3GAL2	6483	-0.620557849	0.245821884	0.866379733	18595
GFM2	84340	-0.643382915	0.22116762	0.864550535	18594
KARS	3735	-1.523906253	-0.660322537	0.863583716	18593
PPP1R14A	94274	-0.532621279	0.330690572	0.86331185	18592

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TSKS	60385	-0.4689732	0.393328528	0.862301728	18591
C1orf189	388701	-0.473581897	0.388012974	0.861594871	18590
FBXO9	26268	-0.486621672	0.373327627	0.859949299	18589
TMEM185B	79134	-0.420942271	0.438961214	0.859903486	18588
RACGAP1	29127	-0.941026145	-0.081305464	0.859720681	18587
PPP4C	5531	-0.781578315	0.078026037	0.859604352	18586
OR10AG1	282770	-0.437782583	0.42110005	0.858882633	18585
TCF23	150921	-0.501343293	0.356750304	0.858093597	18584
SIM1	6492	-0.494943538	0.362921753	0.857865291	18583
OR8G2	26492	-0.502260664	0.355443939	0.857704603	18582
PIM2	11040	-0.512504641	0.34456507	0.857069712	18581
VGLL2	245806	-0.27840028	0.577413397	0.855813677	18580
OR2H1	26716	-0.474979796	0.380467589	0.855447385	18579
CNGA2	1260	-0.266561824	0.587819271	0.854381095	18578
FAM24A	118670	-0.44833235	0.405378621	0.853710971	18577
PRKG1	5592	-0.264268397	0.589440966	0.853709362	18576
SIGLEC15	284266	-0.468885831	0.384386684	0.853272515	18575
CEBPD	1052	-0.378262674	0.474638497	0.852901171	18574
MTHFD2L	441024	-0.410086713	0.442272174	0.852358888	18573
FUZ	80199	-0.472642684	0.379701788	0.852344472	18572
NHP2	55651	-2.009217395	-1.156989054	0.852228342	18571

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
OR2V2	285659	-0.505471463	0.346659759	0.852131223	18570
TRIP13	9319	-0.380665312	0.471350061	0.852015373	18569
RNFT1	51136	-0.583688269	0.267557097	0.851245366	18568
LNK1	84708	-0.419435162	0.431753683	0.851188844	18567
CHRM1	1128	-0.528755215	0.321838822	0.850594036	18566
SLC6A14	11254	-0.282047922	0.568111176	0.850159098	18565
TMEM240	339453	-0.482537186	0.36735889	0.849896076	18564
TFIP11	24144	-0.857392482	-0.007546736	0.849845746	18563
MPP1	4354	-0.32645305	0.522996531	0.849449582	18562
C9orf3	84909	-0.379857152	0.469458084	0.849315236	18561
MRPS24	64951	-1.078522596	-0.230055408	0.848467188	18560
SPA17	53340	-0.093176507	0.755146628	0.848323135	18559
CLK3	1198	-0.392460083	0.454975449	0.847435533	18558
TBX19	9095	-0.49566433	0.35042119	0.84608552	18557
STK19	8859	-0.517113339	0.328235506	0.845348845	18556
PFN4	375189	-0.458925802	0.386053426	0.844979228	18555
PRRT3	285368	-0.493785903	0.351164467	0.84495037	18554
EMB	133418	-0.494419326	0.349295013	0.843714339	18553
DRD5	1816	-0.454928685	0.388733727	0.843662412	18552
MAGEL2	54551	-0.400214053	0.443240687	0.84345474	18551
SASS6	163786	-0.927833475	-0.084877697	0.842955778	18550

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SYT17	51760	-0.382893214	0.459592774	0.842485988	18549
PGAM1	5223	-0.825590285	0.016806838	0.842397123	18548
OPN1MW2	728458	-0.498438285	0.343889364	0.842327649	18547
AAGAB	79719	-0.618985213	0.223341141	0.842326354	18546
AGPAT2	10555	-0.443046546	0.398824271	0.841870817	18545
SDHA	6389	-1.134001704	-0.2921528	0.841848904	18544
PPP2R2B	5521	-0.364698687	0.476552998	0.841251685	18543
TAS2R3	50831	-0.294781906	0.545767827	0.840549733	18542
CT47A2	728090	-0.531354433	0.309022929	0.840377362	18541
CAPN6	827	-0.236004631	0.60423893	0.84024356	18540
SNRNP200	23020	-1.202891903	-0.362719041	0.840172862	18539
FOXRED1	55572	-0.780180416	0.058644534	0.83882495	18538
C10orf113	10529	-0.228774873	0.609982432	0.838757304	18537
PDXP	57026	-0.292597689	0.54610568	0.83870337	18536
PET117	100303755	-1.237489898	-0.39900446	0.838485438	18535
HTATSF1	27336	-1.154795448	-0.31762692	0.837168528	18534
SUSD3	203328	-0.559945831	0.277219695	0.837165526	18533
ANAPC4	29945	-1.193587139	-0.358214334	0.835372805	18532
PMF1	11243	-0.933971125	-0.09892788	0.835043245	18531
C9orf153	389766	-0.279230282	0.554957431	0.834187712	18530
PRDX1	5052	-0.585020641	0.249020226	0.834040867	18529

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
XAGE5	170627	-0.431317301	0.401369431	0.832686732	18528
GTPBP8	29083	-0.868204355	-0.035756115	0.83244824	18527
SLC18A3	6572	-0.351746281	0.48051714	0.832263422	18526
MRPS5	64969	-1.043662495	-0.212061354	0.831601141	18525
FOXJ1	2302	-0.537841557	0.293706924	0.831548481	18524
RNASE9	390443	-0.343817574	0.487251678	0.831069252	18523
RNF114	55905	-0.526243365	0.303910086	0.830153451	18522
IFT172	26160	-0.523513094	0.306297581	0.829810675	18521
TTI1	9675	-1.179433414	-0.350556331	0.828877083	18520
OTUD6B	51633	-0.458139484	0.370714897	0.828854381	18519
SCAMP5	192683	-0.433785466	0.395040317	0.828825783	18518
SEPHS2	22928	-1.505864622	-0.677958467	0.827906156	18517
ZNF439	90594	-0.490400367	0.337470156	0.827870523	18516
PCBP3	54039	-0.523709674	0.304067751	0.827777425	18515
ASPH	444	-0.460192648	0.367584125	0.827776773	18514
TTC30B	150737	-0.316012494	0.511419433	0.827431927	18513
C14orf105	55195	-0.367450801	0.45981801	0.82726881	18512
CDC123	8872	-1.229320927	-0.402090184	0.827230743	18511
AGRN	375790	-0.35545945	0.471710437	0.827169887	18510
PRAMEF8	391002	-0.471812682	0.355308798	0.827121479	18509
RDH14	57665	-0.168676146	0.657957566	0.826633712	18508

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
GLTP	51228	-0.575519298	0.250641921	0.826161218	18507
CRIP1	1396	-0.499486709	0.326163341	0.82565005	18506
SFRP4	6424	-0.339602036	0.485697554	0.82529959	18505
IRAK2	3656	-0.391258764	0.433735754	0.824994518	18504
PRR22	163154	-0.538387611	0.286319204	0.824706815	18503
HMBOX1	79618	-0.215951336	0.60869859	0.824649926	18502
SORL1	6653	-0.338094926	0.485742601	0.823837527	18501
DSG2	1829	-0.339602036	0.483602865	0.823204901	18500
MLF1	4291	-0.443701811	0.379386459	0.82308827	18499
KLHL10	317719	-0.25332547	0.569237353	0.822562823	18498
IMPAD1	54928	-0.291789529	0.530541916	0.822331445	18497
NRG4	145957	-0.336347553	0.485720077	0.82206763	18496
TRERF1	55809	-0.422078064	0.399590072	0.821668136	18495
C9orf163	158055	-0.50734989	0.313617731	0.82096762	18494
PCNA	5111	-1.755695345	-0.934861931	0.820833415	18493
SFXN1	94081	-0.515606229	0.304946169	0.820552398	18492
MED22	6837	-1.403512221	-0.584508311	0.81900391	18491
ANKRA2	57763	-0.5364655	0.282197396	0.818662897	18490
TCEB3CL2	100506888	-0.662254549	0.156103879	0.818358427	18489
MAP3K12	7786	-0.498372758	0.318595432	0.816968191	18488
KHSRP	8570	-0.022281196	0.794495248	0.816776444	18487

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
SSBP3	23648	-0.806543914	0.010111266	0.81665518	18486
FUK	197258	-0.341895463	0.47452588	0.816421343	18485
JMJD6	23210	-0.574099557	0.242285689	0.816385245	18484
PCDHGA5	56110	-0.447065505	0.369250867	0.816316372	18483
MMACHC	25974	-0.662735076	0.153452858	0.816187935	18482
LRRC2	79442	-0.39514667	0.420942385	0.816089055	18481
SRPX	8406	-0.393049822	0.422924456	0.815974278	18480
HKDC1	80201	-0.483913243	0.331546466	0.815459709	18479
SDHAF1	644096	-0.488347203	0.325983152	0.814330356	18478
BFSP1	631	-0.318131184	0.495788099	0.813919283	18477
CXorf40B	541578	-0.404189328	0.409095004	0.813284332	18476
G6PC	2538	-0.451062621	0.361615388	0.81267801	18475
PHLDA2	7262	-0.421663063	0.390873463	0.812536525	18474
PACRGL	133015	-0.351855492	0.458579215	0.810434707	18473
PPP2R4	5524	-1.038551427	-0.228343619	0.810207808	18472
PCDHA2	56146	-0.236922002	0.572390648	0.80931265	18471
MMP19	4327	-0.23569884	0.573471778	0.809170618	18470
UNC13A	23025	-0.294694537	0.51362674	0.808321277	18469
DENND5A	23258	-0.481401394	0.326794	0.808195393	18468
OSBP	5007	-1.134023546	-0.325938105	0.808085441	18467
DNAJC25-GNG10	552891	-0.313478802	0.494571828	0.80805063	18466

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
MRPL53	116540	-0.889260206	-0.081474391	0.807785815	18465
NXN	64359	-0.573335081	0.233343844	0.806678925	18464
FGL1	2267	-0.320075137	0.486575972	0.806651109	18463
PRRT1	80863	-0.331629644	0.474751115	0.806380759	18462
KCNJ18	100134444	-0.658672433	0.14741655	0.806088983	18461
COX19	90639	-0.861826442	-0.055851615	0.805974827	18460
CD83	9308	-0.524364939	0.281026172	0.805391111	18459
C6orf48	50854	-0.479610336	0.325442588	0.805052923	18458
YPEL5	51646	-0.804927593	-0.00075884	0.804168753	18457
KRT77	374454	-0.402114322	0.401955043	0.804069365	18456
TRIM5	85363	-0.350108119	0.453781702	0.803889821	18455
GAREM	64762	-0.196603543	0.607121942	0.803725485	18454
SSR2	6746	-0.38658454	0.416798054	0.803382594	18453
EEFSEC	60678	-0.963414368	-0.160333799	0.803080569	18452
MED7	9443	-1.151715702	-0.348776972	0.80293873	18451
NLRP1	22861	-0.355808925	0.447092211	0.802901136	18450
DUSP28	285193	-0.365397637	0.43664129	0.802038927	18449
C6orf52	347744	-0.33995151	0.462070364	0.802021874	18448
VSTM2B	342865	-0.369984492	0.431933871	0.801918363	18447
OR8A1	390275	-0.507917786	0.293256453	0.801174239	18446
EPHB4	2050	-0.485136404	0.314090725	0.799227129	18445

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
TRAPPC4	51399	-1.41952253	-0.6208613	0.79866123	18444
GPR55	9290	-0.34176441	0.456799856	0.798564266	18443
FSCN1	6624	-0.560186095	0.237353034	0.797539129	18442
UHRF1BP1	54887	-0.452242099	0.34519573	0.797437828	18441
SPATA45	149643	-0.292357425	0.504910131	0.797267556	18440
EIF5A	1984	-0.801935216	-0.005109464	0.796825752	18439
NPHS2	7827	-0.267479195	0.528762557	0.796241752	18438
F2RL1	2150	-0.426031496	0.369949097	0.795980593	18437
RPS8	6202	-1.512439115	-0.716653903	0.795785212	18436
RYR1	6261	-0.504073564	0.291702329	0.795775893	18435
CST1	1469	-0.456523164	0.338979233	0.795502397	18434
LOC101929372	101929372	-0.406744862	0.38837335	0.795118212	18433
RGR	5995	-0.402944324	0.392112257	0.795056582	18432
SRSF12	135295	-0.301290872	0.493558268	0.794849141	18431
C17orf64	124773	-0.546469213	0.248254426	0.794723639	18430
OR4D10	390197	-0.241727279	0.552614983	0.794342261	18429
KIT	3815	-0.331127274	0.462610928	0.793738203	18428
AVP	551	-0.212371404	0.581355016	0.79372642	18427
CCL18	6362	-0.373304502	0.420244155	0.793548657	18426
TMEM246	84302	-0.376755564	0.416640389	0.793395953	18425
DSCAM	1826	-0.397352729	0.395941259	0.793293988	18424

Table A.2: (continued)

Gene	EntrezID	DMSO_beta	PRMT5i_beta	Diff	Rank
MAP2K2	5605	-0.336697027	0.45657462	0.793271648	18423
CGB7	94027	-0.739968984	0.053218613	0.793187598	18422
SORCS2	57537	-0.293165585	0.499504482	0.792670068	18421
DEFB132	400830	-0.577026407	0.214291184	0.791317591	18420
CDKL2	8999	-0.273180001	0.517973783	0.791153784	18419
PRNP	5621	-0.362470786	0.428600387	0.791071174	18418
AURKA	6790	-1.154577026	-0.364273165	0.790303861	18417
SRC	6714	-0.614507568	0.175600252	0.790107821	18416
RAC3	5881	-0.390887447	0.398599036	0.789486483	18415
AGAP3	116988	-0.200489265	0.58883283	0.789322095	18414
C12orf65	91574	-0.61140598	0.177616109	0.789022089	18413
ZSWIM5	57643	-0.545966844	0.243028965	0.788995809	18412
YTHDC1	91746	-0.984426534	-0.19565521	0.788771324	18411
BPIFA3	128861	-0.411746718	0.376683634	0.788430353	18410
TTR	7276	-0.28746478	0.500743277	0.788208056	18409
SEPP1	6414	-0.432889937	0.354858327	0.787748264	18408
ANKFY1	51479	-0.536290763	0.251430245	0.787721008	18407
SMG6	23293	-1.176615774	-0.389431956	0.787183818	18406
ANXA13	312	-0.364938951	0.422203703	0.787142654	18405

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THIS DISSERTATION WAS TYPESET using L^AT_EX, originally developed by Leslie Lamport and based on Donald Knuth's T_EX. A template that can be used to format a PhD thesis with this look and feel has been released under the permissive MIT (X11) license, and can be found online at github.com/suchow/Dissertate or from its author, Jordan Suchow, at suchow@post.harvard.edu.