The Protein Arginine Methyltransferase PRMT5 Regulates Proliferation
and the Expression of MITF and p27Kip1 in Human Melanoma

DISSEMINATION

Presented in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy in the Graduate School of The Ohio State University

by

Courtney Nicholas

Graduate Program in Molecular, Cellular, and Developmental Biology

The Ohio State University

2012

Dissertation Committee:

Gregory B. Lesinski, PhD, Advisor

Jiayuh Lin, PhD

Amanda E. Toland, PhD

Susheela Tridandapani, PhD
Abstract

The protein arginine methyltransferase-5 (PRMT5) enzyme is a Type II arginine methyltransferase that can regulate a variety of cellular functions. We hypothesized that PRMT5 plays a unique role in regulating the growth of human melanoma cells. Immunohistochemical analysis indicated significant upregulation of PRMT5 in human melanocytic nevi (88% of specimens positive for PRMT5), malignant melanomas (90% positive) and metastatic melanomas (88% positive) as compared to normal epidermis (5% of specimens positive for PRMT5; p<0.001, Fisher’s exact test). Furthermore, nuclear PRMT5 was significantly decreased in metastatic melanomas as compared to primary cutaneous melanomas (p<0.001, Wilcoxon rank sum test). Human metastatic melanoma cell lines in culture expressed PRMT5 predominantly in the cytoplasm. PRMT5 was found to be associated with its enzymatic cofactor Mep50, but not associated with STAT3 or cyclin D1. However, histologic examination of tumor xenografts from athymic mice revealed a heterogeneous pattern of nuclear and cytoplasmic PRMT5 expression. siRNA-mediated depletion of PRMT5 inhibited proliferation in a subset of melanoma cell lines, while it accelerated the growth of others. Loss of PRMT5 also led to reduced expression of MITF (microphthalmia-associated transcription factor), a melanocyte-lineage specific oncogene, and increased expression of the cell cycle regulator p27^Kip1. These results represent the first characterization of PRMT5 expression in clinical melanoma specimens and indicate this protein can regulate
melanoma cell growth via pathways involving MITF and p27. Taken together, these data suggest that PRMT5 may play a role in melanocytic proliferation and expression of the major cellular proteins MITF and p27$^{\text{Kip1}}$. 
Dedication

To my mom and dad:

Thank you for guiding me, and at the same time for allowing me to become whoever I wanted to be.
Acknowledgements

The biggest debt of gratitude I owe is to my advisor, Dr. Gregory B. Lesinski. Greg, you took a huge risk by adopting me into your lab. Thank you for believing in me. Your trust has helped me more than you know. An equal amount of thanks go to my director, Dr. David M. Bisaro. Thank you for your support, and for the support of the program, during a time when I didn’t think I deserved it. I will always strive to do my best for the good of science and humanity; this is my promise to you both.

To my friends who saved me when I needed saving: Dr. Oliver Voss, Melissa Crawford, Janet Ann Zinaich, Dr. Lucille Pourcel, Dr. Antje Feller, Maria Isa Casas, Dr. Gwen Philibert, Dr. Bethany Mundy-Bosse, Alena Cristina Jaime-Ramirez, and Dr. Asha Ramanunni. Thank you for being my safety net. I would not have made it without you.

To my grandmother Evelyn, my grandmother Marion and my grandfather Robert; thank you for your love and friendship, and for being the best cheering section that anyone has ever had.

Thank you to all the scientists who helped me grow: Dr. Kenneth Belanger, Dr. Trudy A. Dickneider, Dr. Leonard H. Augenlicht, Dr. John M. Mariadason, Dr. Diego Arango, Dr. Andrea I. Doseff, Dr. Susheela Tridandapani, Dr. Amanda E. Toland, Dr. Jiayuh Lin, Dr. Gustavo Leone, Dr. Mark Parthun, and Christie A. Newland.
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Vita

February 7, 1977 .................................. born; Scranton, Pennsylvania

May 2000 ........................................... B.S. Biology, The University of Scranton

December 2009 ..................................... M.S. Molecular Cellular and Developmental Biology, The Ohio State University

January 2010 to present ......................... Graduate Research Associate, Molecular Cellular and Developmental Biology, The Ohio State University

Selected Publications


Field of Study

Major Field: Molecular, Cellular and Developmental Biology
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>endothelial growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>H4R3</td>
<td>histone H4 arginine 3</td>
</tr>
<tr>
<td>H4R3me2s</td>
<td>symmetric dimethylation of histone H4 at arginine 3</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP-ribose polymerase</td>
</tr>
<tr>
<td>PRMT</td>
<td>protein arginine methyltransferase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoproteins</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TYK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
During development, the human epidermis is derived from neuroectodermal tissue, which eventually gives rise to both skin epithelium and the nervous system. Adult epidermis is derived from pluripotent epithelial cells. In its fully differentiated state it is made up of differentiated keratinocytes in the outermost layer, followed by closely-packed basal epithelial cells bound to extracellular matrix and a population of melanocytes (pigment-producing cells) which vary in number. At the same time during development, mesenchymal cells which infiltrate beneath the epithelium can interact with the cells above to orchestrate the development of sebaceous glands and hair follicles. Finally, beneath the epidermal layer lies the vascularized dermis, which exhibits comparatively low cell density and contains largely connective tissue, extracellular matrix, and vasculature. Pigment-producing melanocytes not only populate the basal epidermal layer of the skin, as well as the base of hair roots and the hair itself, but the pigmented iris and retina of the eye, and certain structures of the inner ear and central nervous system. These cells express the various enzymes necessary to synthesize melanin, a spectrum of various types of chromophores responsible for creating the diverse color tones seen in human skin and hair. These chromophores are manufactured in melanocytes, but then secreted into the keratinocyte layers of the epidermis (Figure 1). Melanin serves to absorb damaging photons from sunlight,
thereby protecting the cells below the epidermis from UV-induced DNA mutations and carcinogenesis.

**Melanoma Skin Cancer**

Melanoma is a cancer arising within the melanocytes of the skin (pigment-producing cells described above). While it is not the most frequently diagnosed skin cancer, it is the most deadly. Melanoma accounts for about 3.6% of newly diagnosed cases of skin cancer, but the majority (>75%) of all skin cancer related deaths are due to metastatic melanoma\(^4\)-\(^5\). In 2012, there are predicted to be 76,250 newly diagnosed cases of melanoma in the United States, with 44,250 of these cases in men and 32,000

![Anatomy of Human Skin](image)

**Figure 1. Anatomy of Human Skin**
in women. There are estimated to be 9,180 deaths from melanoma. In contrast, basal and squamous cell carcinoma (less aggressive, non-melanoma cancers of the skin) account for more than 2 million cases estimated in 2012. Although there has been an increase in detection and diagnosis of melanoma tumors which are <1mm thick, incidence rates of all thicknesses of melanoma tumors (from <1mm to >4mm) have increased from 1996 to 2006. Mortality from primary melanoma skin tumors was estimated to be 1.3%; however, mortality from metastatic disease was 84.5% in 2009.

While very early stage primary melanomas are most often addressed successfully by surgery, advanced stage melanoma is more likely to be accompanied by metastatic disease. Following surgical biopsy of the tissue, the pathology of melanoma is staged in several ways. Clark’s staging and Breslow thickness scores are two means by which the depth of invasion and size (respectively) of the tumor are described. Clark’s staging describes the invasion of the tumor relative to the strata of the normal skin. Clark’s stage I is often termed tumor in situ (Tis), where the tumor is confined to the epidermis (Figure 1). Clark’s stage II involves invasion of the upper papillary dermis, while in stage III the tumor has filled the papillary dermis but not extended to the lower reticular dermis. In Clark’s stage IV, the tumor has invaded the lower reticular dermis and in stage V has invaded the deep subcutaneous tissue. A similar analysis, Breslow thickness, describes the overall size of the tumor in millimeters (mm) regardless of its relative position in layers of the skin. A Breslow score of T1 is assigned to a tumor which is 1mm or less in thickness. T2 describes a tumor between 1.01-2.0 mm, T3 is a tumor between 2.01-4.00 mm thick, and T4 describes a tumor greater than 4.0 mm in thickness. These classification systems, however, do not convey the status of the patient in terms of regional or distal metastases. The TNM (Tumor-Node-Metastasis)
classification system incorporates the Breslow thickness, ulceration status of the primary tumor, and lymph node and distal metastasis data (Table 1)\textsuperscript{11-12}. For example, a patient with a 2.5 mm ulcerated tumor, one regional lymph node positive for micrometastasis, but no metastatic tumors in other organs would have a classification of T\textsubscript{3a}N\textsubscript{1a}M\textsubscript{0}. Values for T, N, and M are explained further in Table 1, and more detailed information on melanoma staging as well as staging for other cancers can be found at the American Joint Committee on Cancer (\url{www.ajcc.org}).
The populations at greatest risk for melanoma-associated morbidity and mortality are non-Hispanic Caucasian men\(^7\), non-Hispanic Caucasians aged 70 and older, people with a history of sun exposure or sunburn, family history of melanoma, and organ transplant patients or other patients whom are immunosuppressed\(^4\). Analysis of all age groups reveals that over all, men have a 1 in 37 chance of developing invasive melanoma, in contrast to women who have a 1 in 56 chance\(^4\). However, women aged 0-39 years are twice as likely to develop an invasive melanoma compared to men of the same age (1 in 360 women versus 1 in 638 men)\(^4\). Organ transplant patients who are receiving immunosuppressive medications exhibit a significantly increased incidence of

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Table 1. TNM clinical/pathological staging system for cutaneous melanoma

<table>
<thead>
<tr>
<th>T (tumor thickness)*</th>
<th>N (node)#</th>
<th>M (metastasis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T(_0)</td>
<td>melanoma in situ</td>
<td>(N_x)</td>
</tr>
<tr>
<td>T(_1)</td>
<td>≤1 mm</td>
<td>(N_0)</td>
</tr>
<tr>
<td>T(_2)</td>
<td>1.01-2.0 mm</td>
<td>(N_1)</td>
</tr>
<tr>
<td>T(_3)</td>
<td>2.01-4.0 mm</td>
<td>(N_2)</td>
</tr>
<tr>
<td>T(_4)</td>
<td>&gt;4 mm</td>
<td>(N_3)</td>
</tr>
</tbody>
</table>

* further classification:  
  a = without ulceration  
  b = with ulceration

# further classification:  
  a = micrometastasis  
  b = macrometastasis (gross extracapsular extension)  
  c = metastasis in transit (intralymphatic)

\(\dagger\) lactate dehydrogenase

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The populations at greatest risk for melanoma-associated morbidity and mortality are non-Hispanic Caucasian men\(^7\), non-Hispanic Caucasians aged 70 and older, people with a history of sun exposure or sunburn, family history of melanoma, and organ transplant patients or other patients whom are immunosuppressed\(^4\). Analysis of all age groups reveals that over all, men have a 1 in 37 chance of developing invasive melanoma, in contrast to women who have a 1 in 56 chance\(^4\). However, women aged 0-39 years are twice as likely to develop an invasive melanoma compared to men of the same age (1 in 360 women versus 1 in 638 men)\(^4\). Organ transplant patients who are receiving immunosuppressive medications exhibit a significantly increased incidence of
melanoma skin cancer, and incidence of melanoma decreased as immunosuppressive therapy was reduced. Furthermore, the risk of melanoma in transplant patients increased with increased sun exposure as well as increased doses of immunodepleting antibody treatments\textsuperscript{13-14}. This suggests that a dysregulated immune system plays a key role in melanoma.

Metastatic melanoma refers to melanoma cells which have spread beyond the primary tumor. The earliest metastases are often detected in the lymphatic system preceding the first draining lymph node (termed an \textit{in-transit metastasis})\textsuperscript{15}. Metastatic cells detected within the first lymph node are classified as local or regional metastases. In the most advanced stage of metastasis, primary tumor cells have moved beyond the lymphatic system to become established in various organ sites, and are termed distal metastases. While the limitations of imaging techniques may bias their detection and quantification, distal metastatic melanoma tumors are most often detected in the skin or subcutaneous structures (13-38\%), lung (18-36\%), liver (14-20\%), brain/CNS (2-20\%), and bone (4-17\%)\textsuperscript{15-18}. Interestingly, data suggest that anatomical location of the primary tumor (for example, head/neck versus trunk or lower extremities) may determine the likelihood and location of distal metastatic disease\textsuperscript{15,19-21}.

The standard of care for primary melanoma is surgery, while for metastatic disease, the chemotherapeutic agent dacarbazine (a DNA-alkylating agent) is most often used\textsuperscript{22}. Recent advancements in treatment have provided some hope for patients; tumors which have an activating mutation in the \textit{BRAF} gene are candidates for targeted \textit{BRAF} inhibitor therapies such as Vemurafenib (Zelboraf\textsuperscript{TM}; Plexxicon/Hoffman-LaRoche). At the same time, alternative strategies such as Ipilimumab (Yervoy\textsuperscript{TM}; Bristol-Meyers/Squibb) promote immune-mediated anti-tumor response through
reactivation of cytotoxic T cells\textsuperscript{23-24} (Table 2). However, due to progressive metastatic disease and tumors whose \textit{BRAF} status is not amenable to inhibitor therapy, the five-year survival rate is approximately 15\%\textsuperscript{4}. Therefore, there is a concerted effort to identify novel molecular targets and explore alternative treatment strategies for metastatic melanoma beyond traditional chemotherapies, and those with clinical benefit irrespective of tumor genetic profile.

Table 2. Clinical therapies currently in use against metastatic melanoma.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Trade Name</th>
<th>Manufacturer</th>
<th>FDA approval</th>
<th>Mechanism</th>
<th>Cancer Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vemurafenib</td>
<td>Zeolcoraf</td>
<td>Hoffman-LaRoche</td>
<td>August 2011</td>
<td>Inhibition of BRAF activity</td>
<td>\textit{BRAF}\textsubscript{V600E} melanoma</td>
</tr>
<tr>
<td>Ipilimumab</td>
<td>Yervoy</td>
<td>Bristol-Meyers/Squibb</td>
<td>May 2011</td>
<td>T cell re-activation</td>
<td>melanoma</td>
</tr>
<tr>
<td>Interleukin-2 (IL-2)</td>
<td>Aldesleukin/Proleukin</td>
<td>Prometheus</td>
<td>1998</td>
<td>NK and T cell activation</td>
<td>melanoma, renal cell carcinoma</td>
</tr>
<tr>
<td>Interferon-α2b</td>
<td>Pegasis</td>
<td>Hoffman-LaRoche</td>
<td>1995</td>
<td>NK and T cell activation</td>
<td>melanoma</td>
</tr>
<tr>
<td>Dacarbazone</td>
<td>DTIC-Dome</td>
<td>Bayer</td>
<td>1975</td>
<td>purine analog/DNA damage</td>
<td>melanoma</td>
</tr>
</tbody>
</table>

\textit{MITF – an Essential Melanocyte Transcription Factor and Oncogene}

Maturation of melanocytes is a complex process. However, one major protein has been well-characterized to be essential to this differentiation process. The MITF (microphthalmia-associated transcription factor) protein was first identified through mutation analysis in 1942 by Paula Hertwig\textsuperscript{25} and cloned in 1993\textsuperscript{26}. The first observation of mutations at this locus showed its involvement in eye development; mice with mutations at that locus had small, undeveloped and nonfunctional eyes. Thus, MITF was named for this phenotype, the name microphthalmia being Greek for ‘small eye’.
These mice also demonstrated a lack of coat color as well as defects in the hair cells of the ear\textsuperscript{25}. Further work with this protein demonstrates that it is essential for melanocyte development\textsuperscript{27}. MITF is a basic-helix-loop-helix protein which binds to DNA promoters and directs gene expression. Phosphorylation of MITF allows it to bind and recruit other transcription factors and chromatin modifiers to gene promoters, allowing gene expression to take place\textsuperscript{28}. Mutations in the $MITF$ gene in mice lead to absence of coat color, small undeveloped eyes, and deafness. In humans, mutations in $MITF$ can also lead to pigmentation disorders and deafness (called Waardenburg syndrome)\textsuperscript{29}.

MITF protein activation is mediated partly by phosphorylation, and driven by several proteins including ligand binding to the c-Kit receptor\textsuperscript{30}. Expression of MITF is regulated through the Wnt/$\beta$-catenin pathway, among others\textsuperscript{28,31}. It has been characterized as an oncogene. In 2005, a gene duplication event was discovered in melanoma, resulting in increased copy number (and increased dosage) of $MITF$\textsuperscript{32}. In this seminal study, increased dosage of $MITF$ was seen in 5/49 (10.2\%) of primary melanomas, 34/192 (17.7\%) of metastatic melanomas, but in 0/19 (0\%) of melanocytic nevi examined. Furthermore, increased $MITF$ copy number inversely correlated with survival\textsuperscript{32}. $MITF$ amplification appears to promote survival of cells, conferring independence from or hyper-reaction to MITF-related growth signals.

\textit{Dysregulated Molecular Pathways in Cancer and Melanoma}

Malignant transformation of any cell type is mediated by many different types of changes. In general, these changes can be categorized as follows: dysregulation of cell division, hypersensitivity to or independence from growth signals, insensitivity to apoptotic signals, inappropriate response to DNA damage, inappropriate remodeling of
the microenvironment, anchorage-independent growth, and ability to evade or inactivate the immune system\textsuperscript{33}. In melanoma, several common mutations have been characterized which have the potential to impact each of these cellular processes.

The most frequently detected mutations in melanoma are found in proteins which regulate the cell cycle (Figure 2). Periodic expression of two types of proteins – cyclins and cyclin-dependent kinases – allows cells to progress through two growth phases, DNA synthesis, and mitosis. Throughout this cycle are several checkpoints, during which cells survey DNA for mutations. As a result, cells have the ‘option’ of repairing these lesions and proceeding through cell division (if the lesions are reparable), or if the mutations are too severe, cells can undergo apoptosis to prevent these mutations from being retained in the subsequent daughter cells\textsuperscript{34}. The tumor suppressor protein p53 is one of the major mediators of cell cycle arrest and DNA repair. p53 controls the expression of several proteins called cyclin dependent kinase inhibitors (CKIs), which bind and inhibit various cyclin dependent kinases and prevent cells from progressing through the cell cycle\textsuperscript{35}. p16\textsuperscript{INK4a} is the most frequent CKI found to be mutated or aberrantly expressed in melanoma. While the exact nature of p16\textsuperscript{INK4a} alterations in melanoma is unclear, there is longstanding historical evidence of inactivating mutations, as well as suppression of p16\textsuperscript{INK4a} expression through promoter methylation\textsuperscript{36-37}. Together, these result in dysregulated cell cycle progression in melanoma. As further evidence of the important role of p16\textsuperscript{INK4a} in melanocyte proliferation and melanoma, it is known that the MITF transcription factor, which is instrumental in melanocyte development, is also a major regulator of p16\textsuperscript{INK4a} expression\textsuperscript{37}. 
However, a more clinically significant understanding of aberrant pathways in melanoma lies in the discovery of activating mutations in the RAS/RAF/MEK pathway\textsuperscript{38-39}. Similar to the Jak/STAT pathway, the RAS/RAF/MEK pathway translates signals from the cell surface to the nucleus, ultimately affecting gene expression and major cellular processes. The RAS protein is a membrane-associated protein which, following activation of cell-surface RTKs, becomes bound to GTP and is then able to bind and activate the serine/threonine kinase B/CRAF. RAF mediates activation of downstream targets MEK and ERK/MAPK through phosphorylation, and these proteins activate transcription factors such as Jun, Fos, and Myc, to upregulate genes involved in

Figure 2. The Eukaryotic Cell Cycle
proliferation and other major cellular processes\textsuperscript{40}. Under normal conditions, GTP is hydrolyzed to GDP, RAF dissociates from RAS, and the downstream pathway becomes inactive. However, it has been reported in several studies that 30-70\% of melanoma tumors contain the \textit{BRAF}^{V600E} activating point mutation, with the largest clinical trial demonstrating the \textit{BRAF}^{V600E} mutation in 50\% of tumors\textsuperscript{41-44}. The \textit{BRAF}^{V600E} mutation results in constitutive phosphorylation and activation of MEK, independent of receptor activation\textsuperscript{45}. Hyperactivation of this pathway results in increased proliferation. The RAS/RAF/MEK pathway represents another set of potential therapeutic targets. Generic RTK inhibitors such as sunitinib, gefitinib, and erlotinib have resulted in clinical response in the treatment of non-small cell lung, gastrointestinal, breast, and renal cancers\textsuperscript{46-47}. More recently, the first BRAF inhibitor, vemurafenib, has been approved by the FDA in the United States for use in the treatment of BRAF mutant metastatic melanoma\textsuperscript{48}. While vemurafenib extends survival by approximately 5 months, there is a high incidence of acquired resistance to the drug, possibly due to activation of alternative pathways. A similar limitation lies in the fact that vemurafenib is not appropriate for use in \textit{BRAF} wild-type tumors, due to acceleration of tumor growth by a yet poorly understood mechanism that appears to involve trans-activation of CRAF\textsuperscript{49}.

The Jak/STAT signal transduction pathway serves to translate growth signals from the cell surface receptors to the nucleus, and is aberrantly activated in cancer\textsuperscript{50-54}. In normal cells, transmembrane receptors such as interferon and interleukin receptors have intracellular domains which are associated with several different types of receptor tyrosine kinases (RTKs), such as the Janus kinases (Jak) or TYK proteins. These receptors dimerize upon ligand binding, bringing together two Jak monomers which then undergo transphosphorylation of each other, as well as the intracellular domains of the
receptor. Phosphorylation events result in recruitment of various Signal Transducer and Activator of Transcription (STAT) proteins to the receptor, which in turn are phosphorylated and then dimerize. Subsequently, activated STAT dimers translocate to the nucleus where they then interact with DNA and regulate expression of genes involved in proliferation and inflammation\textsuperscript{55}. STAT proteins are a family of six transcription factors with often divergent effects on cell biology. For example, STAT1 homodimer and STAT1/2 heterodimers are associated with an anti-proliferative effect, and are largely responsible for regulating expression of immunomodulatory cytokines and chemokines, and promoting anti-tumor immunity\textsuperscript{56}. In contrast, STAT3 activation (as either STAT1/3 heterodimers or STAT3 homodimers) is associated with increased proliferation, survival, angiogenesis, and immunosuppression. In melanoma, as well as in other types of cancers, the STAT3 pathway is constitutively activated through kinase hyperactivity and constitutively phosphorylated STAT3 dimers\textsuperscript{57}. As novel treatment options such as immunotherapy are pursued, the STAT3 pathway has become a target of interest in melanoma and other cancers. To allow for proper anti-tumor activity by the immune system, molecular inhibitors which specifically target STAT3 but not STAT1 are being sought\textsuperscript{58-59}.

\textit{Epigenetic and Post-Translational Modifications in Cancer}

Amino acid sequence is only a partial determinant of the final conformation and function of a protein. Proteins can fold, multimerize, or be enzymatically cleaved, either to perform their intended functions, or to be inactivated. Chemical groups can also be added to specific amino-acid side chains in order to mediate function. Molecules such as phospho-, methyl-, and acetyl- groups, or short peptides such as ubiquitin and SUMO
are just a few of an ever-growing family of post-translational modifications which can mediate protein function\textsuperscript{60-62}.

Similarly, there are many different families of proteins which are subject to these types of modifications. The same modification on two different proteins can have dramatically different effects on each. For example, many kinases including the BRAF kinase (mentioned previously) are activated upon phosphorylation. However, phosphorylation of the IκB (inhibitor of kappa B) protein leads to its ubiquitination and degradation\textsuperscript{63}. Of particular interest in cancer are proteins which have been shown to perform post-translational modifications on nucleosomal histones, structural proteins which function in the nucleus to spool and organize DNA, and to regulate access to gene sequences. Eight histones assemble to form a positively-charged hetero-octomer (called a nucleosome), in a conformation which allows free access to each histone’s amino terminal tail, and around which DNA (with its strongly negative backbone) is wound\textsuperscript{64}. These amino terminal histone tails are subject to several different types of post-translational modifications which alter the charge of the nucleosome and can thus change the strength of its association with DNA. Histone acetyltransferases are enzymes which acetylate specific lysine residues on the histone tail, conferring a more neutral charge on the nucleosome, and allowing it to be repositioned or removed. These alterations allow transcriptional complexes to access DNA promoters and express genes. Furthermore, the specific pattern of acetylation on histones appears to regulate recruitment of these transcriptional complexes\textsuperscript{60}. Conversely, histone deacetylases remove these acetyl groups and restore the nucleosome’s tight association with DNA.

Methylation (CH\textsubscript{3}) of histones is another mechanism by which access to genes is regulated in a heritable manner. CH\textsubscript{3} groups are attached to lysine or arginine residues
in different configurations, which have divergent effects on gene expression. Recent discoveries have revealed that specific types of histone methylation are a prerequisite for DNA methylation, a major mechanism for gene silencing and a modification maintained through cell division and copied onto the DNA of subsequent daughter cells. Methylation, however, is not a modification which is limited to nucleosomal histones. Many proteins which are soluble and cytoplasmic, as well as proteins which shuttle between the nucleus and the cytoplasm, have been shown to be targets of lysine and arginine methylation. For example, Jak2, STAT1, p53, and myelin basic protein (MBP) are examples of proteins shown to be methylated. This methylation can lead to altered protein function or proteasome-mediated degradation.

**The Protein Arginine Methyltransferase Family of Enzymes**

Protein arginine methyltransferases (PRMTs) are a family of eleven enzymes which mediate the methylation of various cellular proteins. These enzymes catalyze the methylation of nitrogen groups exclusively on the amino acid arginine. Categorized based on sequence homology, mammalian PRMTs appear to be conserved through evolution, having orthologs in *drosophila*, *S. cerevisiae*, *C. elegans*, *Arabidopsis thaliana*, amoeba, and *plasmodium falciparum* (malaria). The family members most highly conserved across species are PRMT1 and PRMT5. All family members are categorized according to their conserved sequence homology; each contains at least one catalytic domain which associates with the methyl donor, S-adenosyl-methionine (SAM). It is important to note that, while these proteins have been categorized according to sequence homology, some family members (PRMT10 and PRMT11) have not yet been shown to actually participate in methyltransferase reactions in vivo.
Interestingly, these same proteins which have not yet been associated with methyltransferase activity also appear to have gene sequences which diverged very early on from the rest of the PRMT family. In a graphical representation of sequence analyses performed by Krause et al., public databases were mined for PRMT gene sequences and then subjected to sequence alignment using the ClustalX software package and associated Neighbor-Joining plot function\(^{69-70}\) (Figure 3). Further sequence analysis reveals that the majority of the Type I PRMT enzymes (PRMT 1, 2, 3, 4, 6, 8) represent an evolutionarily younger subgroup, evolving after the emergence of a gene duplication event which conferred two catalytic domains to PRMT7 and PRMT10, and well after the divergence of PRMT\(^{69}\) (Figure 3).

The positively charged side chain of the amino acid arginine allows for two potential covalent modifications (Figure 4). Both of the terminal (ω) nitrogen atoms represent a potential target for stepwise mono- and subsequent dimethylation. Due to electron resonance, mono-methylation can occur on either of the two ω–nitrogen atoms with equal stereochemistry. However, dimethylation reactions can be catalyzed in one of two ways – asymmetric methylation (di-ω-\(N,N\)-dimethylarginine), with two CH\(_3\) groups placed on the same nitrogen atom, or symmetric dimethylation (di-ω-\(N,N'\)-dimethylarginine), in which a CH\(_3\) group is placed on each of the two terminal nitrogen atoms. PRMT enzymes have been separated into two distinct families, based on type of methylation modification which they catalyze. In vitro, Type I enzymes (PRMT 1, 2, 3, 4, 6, 8) catalyze an asymmetric dimethylation of the arginine side chain, while Type II enzymes (PRMT5, 7, 9) are able to catalyze symmetric dimethylation reactions\(^{69,71}\) (Figure 4). Interestingly, these two types of dimethylation can often occur on the same protein, yet exert quite different effects. For example, asymmetric dimethylation of
nucleosomal histones often results in an upregulation of gene expression at that locus. In contrast, symmetric dimethylation of histones has been linked to silencing of gene expression.

While there are many unanswered questions surrounding the complete functions of the PRMT family, several family members have been implicated in major cellular signaling networks. PRMT1, one of the smallest PRMTs, is characterized as a Type I methyltransferase. PRMT1 was identified in a yeast two-hybrid screen, to bind the STAT1 transcription factor. STAT1 was found to be methylated on Arg31, independent of Tyr701 phosphorylation, the characteristic phospho-modification which leads to STAT1 activation\textsuperscript{68}. Site-directed mutagenesis of Arg31 to Ala (mimicking the addition of a methyl group canceling out arginine’s positive charge) resulted in increased expression of STAT1 target genes. Conversely, inhibition of methylation through treatment with MTA (methylthioadenosine, a nonspecific methyltransferase inhibitor) resulted in decreased expression of STAT1 target genes and increased association between STAT1 and its inhibitor protein, PIAS (protein inhibitor of activated STATs)\textsuperscript{68}. These data demonstrate that PRMT1 is a major factor in regulating the STAT1 protein, which mediates cell proliferation and immune response as described previously.

The scope of work presented in this document will focus on the biological and mechanistic features of one particular family member, PRMT5. This enzyme has generated much interest in the past few years, due to its possible involvement in several types of cancer. While the most well characterized feature of this family of enzymes is related to association with chromatin and involvement in gene expression, the data presented here suggests alternative functions for PRMT5 in the cytoplasm of the cell.
Figure 3. Sequence divergence of PRMT5 proteins, their functional domains, and relative sizes.
The Type II Methyltransferase Enzyme PRMT5

The human PRMT5 gene is found on chromosome 14q11.2. It is one of only two PRMT proteins which appear to be consistently found from yeast evolutionarily upwards through molds, fungi, protozoa, insecta, and vertebrata\(^\text{69}\). Though PRMT5 has been found in both the nucleus and cytoplasm of cells, there does not appear to be any known signal sequence (nuclear localization sequence; NLS) which would target this protein to the nucleus on its own. The PRMT5 protein performs monomethylation, and subsequent symmetric dimethylation of arginine residues, within peptide regions that are rich with arginine and glycine (RG) repeats\(^\text{71-72}\).
It is still not clear what transcription factors regulate the expression of PRMT5. In *arabidopsis thaliana*, for example, the expression of PRMT5 is connected to Earth’s dark/light cycle and seems in turn to regulate many clock genes\(^73\). In vertebrates, it has been suggested based on sequence elements in the *PRMT5* promoter that its expression may be regulated by STAT3 and cMyc (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PRMT5), however, no definitive evidence yet exists. Two independent groups have observed a post-transcriptional mechanism of regulation, either through degradation of mRNA or blockade of translation. In 2007, Pal et al. showed in mantle cell lymphoma that miR92b/96 bind to a sequence in the 3’ UTR of *PRMT5*, and that downregulation of miR92b/96 results in increased expression of *PRMT5* and subsequent increase in H4R3me2s\(^74\). The PRMT5 protein functions as a homodimer, and has also been found in the cell as a homo-oligomer\(^67\). It is not yet known whether the stability of the PRMT5 protein is affected under any circumstances by post-translational modifications including phosphorylation, ubiquitination, etc.

*The Role of PRMT5 in the Cytoplasm*

The earliest functional studies of this protein were performed with homologs found in *S. pombe*, Skb1, and *S. cerevisiae*, Hsl7p\(^75-76\). PRMT5 was first characterized in 1999 in a yeast two-hybrid screen as a cytoplasmic protein which bound to the Jak2 kinase. Sequence comparison revealed a mammalian homolog which was then named JBP1 (Jak Binding Protein 1)\(^67\). In 2001, several groups described PRMT5’s role in another cytoplasmic activity, the regulation of mRNA splicing via the spliceosome, following its transcription in the nucleus. PRMT5 was found in a protein complex called the methylosome, which contains along with PRMT5, a WD repeat-binding protein called...
Mep50 (methylosome protein 50 kDa) and pICln (nucleotide-sensitive inducer of chloride current protein)\textsuperscript{76-78}. PRMT5, as part of this methylosome, associates with and methylates Sm proteins D1 and D3, components of another essential cell complex, the spliceosome. Furthermore, only unmethylated D1 and D3 were able to associate with PRMT5, and this methylation event is essential for the association of D1 and D3 with the SMN (survival of motor neuron) protein, a trimeric association which is essential for spliceosome assembly and function\textsuperscript{79}.

It was not until seven years later that PRMT5 was discovered to interact with and modulate activity of the tumor suppressor protein p53\textsuperscript{66}. Work done in 2008 by Jansson et al. demonstrates that PRMT5 is recruited by phospho-STRAP to p53 in response to DNA damage. In this report, PRMT5 methylates p53 at residues Arg333, Arg335, and Arg337, which appear to be part of p53’s oligomerization and intracellular localization domain\textsuperscript{80-81}. Depletion of PRMT5 appears to alter p53’s promoter binding specificity, and leads to apoptosis. Protein levels of PRMT5 and p53 also appear to be directly related; siRNA mediated depletion of PRMT5 resulted in lower levels of p53 protein irrespective of DNA damage. Ectopic expression of PRMT5 results in increased p53 protein, but this increase is entirely dependent on enzymatic activity of PRMT5. Furthermore, it appears that methylation of these residues may be required for efficient nuclear import of p53, and that these methylation events reduce the stability of the p53 oligomer\textsuperscript{66}. Many of these observations were confirmed in breast cancer cells, in studies from another laboratory around the same time\textsuperscript{82}. Thus, PRMT5 plays a large role in the cellular response to DNA damage.

Two other cytoplasmic targets of PRMT5 are Fen1 (Flap endonuclease 1) and TRAIL (TNF-related apoptosis inducing ligand)\textsuperscript{83-84}. Fen1 is a protein which binds to
PCNA (proliferating cell nuclear antigen) and recruits it to replication forks in dividing cells. Fen1 is also a methyl target of PRMT5; the majority of methylated Fen1 appears in the cytoplasm. Methylation of Fen1 is required in order to allow its interaction with PCNA, and appears to be a molecular switch which controls its interaction with PCNA. Namely, addition of a methyl group abrogates the phosphorylation of a nearby serine. In cells depleted of PRMT5, Fen1 is prematurely phosphorylated, fails to associate with PCNA, and both proteins fail to localize to replication forks83. Since DNA replication is hindered, the rate of mutations is increased, and these mutations persist in the daughter cells.

The TRAIL receptor and its associated signaling pathway are involved in the apoptotic response to extrinsic death signals. Interestingly, it appears that only normal hepatic cells, some hematopoietic cell lineages, and tumor cells from various tissues express the TRAIL receptors DR4 (death receptor 4) and DR5. Though ligand binding of DR4/DR5 receptors activates downstream caspases, it can also subsequently activate anti-apoptotic pathways such as the p38/MAPK and NFκB pathways. In 2009, Tanaka et al. discovered that PRMT5 associates with the intracellular Fas-associated death domain (FADD) of the TRAIL receptor, and is ultimately involved in mediating cellular resistance to TRAIL-induced apoptosis through activation of NFκB. Depletion of PRMT5 via siRNA resulted in decreased activation of IKK and NFκB and re-sensitized cells to TRAIL-mediated apoptosis84.

The targets and pathways mentioned thus far are clearly implicated in the basic cellular functions which are known to be dysregulated in cancer. However, though the characterization of PRMT5 is yet in its infancy, there are several reports which clearly and directly implicate PRMT5 in several human cancers. The role of PRMT5 in
leukemia, lymphoma, breast, testicular, and melanoma skin cancer has been documented, and its interactions with relevant molecules such as cyclin D1, PCD4, and CRAF will be described in subsequent paragraphs.

*The Role of PRMT5 in the Nucleus*

PRMT5 plays a number of different roles in interactions with nuclear proteins. It was shown to associate with the cyclin E1 promoter, as part of the CERC (cyclin E1 repressor complex) in NIH3T3 fibroblasts, and co-localize with histone H4 methylated at arginine 3 (R3), thereby regulating expression of cyclin E1\(^85\). PRMT5 is often thought of primarily as a histone methyltransferase, as it appears to symmetrically dimethylate the histone tails of H2A, H3, and H4 consistently at predicted residues\(^67,71,86-87\). In 2005, experiments showed that methylated proteins associated with the IL2 (interleukin 2) promoter in anti-CD3-activated T lymphocytes, and that depletion of PRMT5 in T lymphocytes resulted in decreased expression of IL2 following CD3-mediated activation\(^88\). Work done in NIH3T3 fibroblasts demonstrated a mechanism by which PRMT5 regulates gene expression. BRG1 and BRM proteins, part of the human SWI/SNF transcription regulation complex, associate with PRMT5 and cooperate to target histone H3 at arginine 8 (H3R8) and H4R3. The functional consequence of this reaction is to suppress expression of the ST7 and NM23 tumor suppressor genes through direct interaction with histones within their DNA promoters\(^87\). HDAC (histone deacetylase) activity may also be involved, as methylation appears to be more efficient on hypoacetylated than hyperacetylated histones\(^86\).

In 2010 Prmt5 was demonstrated in the mouse to be essential for embryonic development\(^89\). Prmt5 is detected in mouse primordial germ cells at embryonic day
E7.5, where it binds with Blimp1 and its associated HDACs\textsuperscript{89-91}. The Prmt5/Blimp1 complex appears in the nucleus from E7.5-11.5, where it symmetrically dimethylates histones H2A and H4 at arginine 3 (H2A/H4R3me2s)\textsuperscript{90}. However, \textit{in vitro} experiments suggest a major role for Prmt5 in maintaining stem cell pluripotency. Treatment of terminally differentiated mouse germ cells with Trichostatin A (a small molecule HDAC inhibitor) or bFGF (basic fibroblast growth factor) results in a phenotypic shift from a unipotent state, back to a pluripotent state. At the same time, Blimp1 is rapidly downregulated and Prmt5 translocates from nucleus to cytoplasm, where it is then free to bind other substrates. This correlation between the translocation of Prmt5 and the switch from unipotency to pluripotency suggests that cytoplasmic Prmt5 has an essential cytoplasmic role in maintaining pluripotency\textsuperscript{89,91}. Furthermore, those same studies show that Prmt5 appears to methylate newly translated histone H2A in the cytoplasm, prior to H2A's translocation to the nucleus and deposition in nucleosomes\textsuperscript{91}. This certainly suggests a novel mechanism by which Prmt5 can regulate gene expression without being present in the nucleus of the cell.

Thus, PRMT5 appears to be a multipurpose protein, with roles in both cytoplasmic and nuclear cellular processes. This diverse character further suggests that dysregulation of PRMT5 could certainly have a profound impact on cell biology, and suggests PRMT5 as a possible contributor to malignant transformation.

\textit{PRMT5 in Cancer}

Despite PRMT5’s apparent involvement in many fundamental cellular processes, understanding of this protein’s role in specific cancers is still incomplete. However, this work has advanced rapidly in recent years. Indeed, PRMT5 has been implicated in the
oncogenic transformation of and cancer progression in several different cell types\textsuperscript{92-96} (summarized in Table 3).

In studies conducted almost simultaneously by different laboratories, PRMT5 was implicated in both testicular cancer and myeloproliferative cancer\textsuperscript{74,97}. In 2007, PRMT5 was reported to associate with the androgen receptor (AR) related protein p44, and was demonstrated to be expressed in both normal testicular tissue as well as in testicular tumors. However, there was a pronounced difference in subcellular localization between tumor and normal tissue. PRMT5 was mostly nuclear in normal testicular cells, but in testicular tumor cells PRMT5 was constrained largely in the cytoplasm. The authors speculated that the presence of PRMT5 and p44 in the nucleus may have an antiproliferative effect in adult tissue. Analyses of normal fetal and adult tissue, as well as tumor tissue suggest that the cytoplasmic targets of PRMT5 and p44 may be unrelated to AR, due to the fact that often, PRMT5 and p44 are cytoplasmic while the AR is nuclear. Interestingly, p44 is reported to be a subunit of the SMN complex mentioned previously\textsuperscript{97}. 
PRMT5’s role in myeloproliferative disorders was also revealed during this time. In 2007, Pal and colleagues built upon substantial previously published work to demonstrate that PRMT5 is upregulated in Burkitt’s lymphoma, mantle cell lymphoma, Epstein-Barr Virus-transformed lymphoma, and leukemia, compared to normal B lymphocytes. Along with this upregulation, there is an increase in methylation of histones H2AR3 and H4R3, as well as a decreased expression of several tumor suppressor genes including ST7 (suppressor of tumorigenicity 7) and NM23-H1 due to PRMT5’s nuclear localization in lymphocytic cells. Through several well-executed experiments testing the stability of PRMT5 mRNA and protein, it was discovered that

<table>
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<tr>
<th>Cancer Type</th>
<th>Features</th>
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<tr>
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<td>overexpressed; nuclear</td>
</tr>
<tr>
<td>mantle cell lymphoma</td>
<td>overexpressed; nuclear; suppresses expression of tumor suppressor genes ST7 and NM23-H1; regulated by miRs (miRs downregulated in lymphoma)</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>overexpressed; nuclear; suppresses expression of tumor suppressor genes ST7 and NM23-H1</td>
</tr>
<tr>
<td>testicular</td>
<td>present but restricted to cytoplasm in tumor (nuclear in normal testicular tissue)</td>
</tr>
<tr>
<td>osteosarcoma</td>
<td>PRMT5 associates with COPR5 in the nucleus, to suppress expression of CCNE1 (cyclin E1)</td>
</tr>
<tr>
<td>breast</td>
<td>binds PDCD4; upregulation of PRMT5 increases proliferation; low PRMT5 correlates with higher 20-yr survival</td>
</tr>
<tr>
<td>melanoma</td>
<td>binds CRAF in PC12, COS-7 cells; modulates ERK pathway in stimulated melanoma cells with BRAF WT genotype</td>
</tr>
<tr>
<td>lung</td>
<td>elevated in lung adenocarcinoma, small cell lung cancer, and lung squamous cell carcinoma; PRMT5 depletion leads to cell cycle arrest and loss of expression of FGFR1/3/4 in A549 cells</td>
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Table 3. Brief summary of PRMT5 characterization in several cancers

The Methyltransferase PRMT5 in Cancer

<table>
<thead>
<tr>
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<td>Liang et al J Urol 2007</td>
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<td>Lacroix et al EMBO 2008</td>
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<td>Powers et al Cancer Res 2011</td>
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<td>melanoma</td>
<td>binds CRAF in PC12, COS-7 cells; modulates ERK pathway in stimulated melanoma cells with BRAF WT genotype</td>
<td>Andreu-Perez et al Science Sig 2011</td>
</tr>
<tr>
<td>lung</td>
<td>elevated in lung adenocarcinoma, small cell lung cancer, and lung squamous cell carcinoma; PRMT5 depletion leads to cell cycle arrest and loss of expression of FGFR1/3/4 in A549 cells</td>
<td>Gu et al Biochem J 2012</td>
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</table>
PRMT5 expression in normal B lymphocytes is post-transcriptionally regulated via microRNAs miR92b/96. In line with this observation, it was further noted that miR92b/96 is significantly downregulated in mantle cell lymphoma, resulting in increased PRMT5 protein translation and silencing of tumor suppressor genes through methylation.

Subsequent work with PRMT5 revealed that this enzyme was involved in the biology of osteosarcoma. PRMT5 was shown to be present in the nucleus of human osteosarcoma cells in vitro, and also shown to associate with the cooperator of PRMT5 protein (COPR5). The association between PRMT5 and COPR5 result in suppression of the expression of the cell cycle regulatory gene cyclin E1 (CCNE1).

PRMT5 has also been studied in mouse models of lymphoma, a cancer which is driven largely by excess stability and activity of the cyclin D1 protein. In 2010 PRMT5 was found to bind to both wild-type and mutated versions of the cyclin D1 protein. Cyclin D1 complexed with CDK4 (cyclin-dependent kinase 4) was able to phosphorylate Mep50. This hyperphosphorylated Mep50 protein in turn leads to increased PRMT5 activity, which in this model was measured by change in expression of the CUL4 gene. This work again highlights a potential nuclear role for PRMT5 in this type of cancer. Suppression of CUL4 can lead to increased activity of the cyclin D1/CDK4 complex, resulting in increased proliferation.

Recent discoveries of PRMT5 and its role in cancer have been very preliminary studies in human breast and melanoma skin cancer. In 2011, Powers et al. described their work with PRMT5 and PDCD4, a protein which behaves like a tumor suppressor and correlates with increased survival rates in patients. PRMT5 appears to bind and methylate the PDCD4 (programmed cell death 4) protein in a mouse orthotopic model using human breast cancer cells (MCF7 cell line). MCF7 cells expressing exogenous
PRMT5 and PDCD4 at the same time grew faster in comparison to cells expressing either PRMT5 or PDCD4 alone. Though PRMT5-mediated methylation of PDCD4 did not appear to affect PDCD4’s stability or downstream interactions, quantification of PRMT5 and PDCD4 expression in human breast cancer patients showed the highest 20-year survival rate in patients with high PDCD4 and low PRMT5. Thus, these two proteins may be useful prognostic markers in human breast cancer\textsuperscript{95}.

PRMT5’s role in melanoma was investigated for the first time by Andreu-Perez \textit{et al.} in 2011. It was demonstrated that growth factor-stimulated melanoma cell lines treated with a pan-methyltransferase inhibitor showed elevated activation of the MEK/ERK signaling pathway and a subsequent switch from proliferation to differentiation. Initial experiments done with nonspecific methyltransferase inhibitory drugs showed that methyltransferase activity suppresses ERK (extracellular signal-related kinase) signaling in melanoma cells, and that siRNA mediated depletion of PRMT5 results in increased pathway activation in response to stimulation with hepatocyte, endothelial, and fibroblast growth factors (HGF, EGF, FGF)\textsuperscript{93}. A physical interaction between CRAF and PRMT5 was also shown in PC12 and Cos-7 cells. However, this physical interaction between PRMT5 and CRAF was not demonstrated in unstimulated melanoma cells, or in \textit{BRAF} wild-type melanoma cells regardless of stimulation. Thus, there is still much work to be done in order to understand PRMT5’s role in melanoma.

Finally, two recent reports have demonstrated elevated PRMT5 in several lung cancers. Gu, \textit{et al.} demonstrates that PRMT5 is elevated, but largely cytoplasmic, in lung adenocarcinoma, small cell lung cancer, and lung squamous cell carcinoma. Interestingly, despite PRMT5’s cytoplasmic localization in these cancers, the authors
also show a loss of fibroblast growth factor receptors 1, 3, and 4 (FGF1, 3, 4) following siRNA mediated PRMT5 depletion\textsuperscript{99}. Depletion of PRMT5 in the A549 lung carcinoma cell line also resulted in cell cycle arrest in G1. Interestingly, overexpression of an enzymatically inactive, siRNA insensitive PRMT5 construct and concurrent siRNA mediated depletion of endogenous (wild-type) PRMT5 also resulted in cell cycle arrest, suggesting that the methyltransferase activity is necessary to promote proliferation in lung carcinoma cells\textsuperscript{99}.

The previous studies of PRMT5, described above, suggest that PRMT5 behaves consistently as an oncoprotein, associated with initiation, maintenance, or progression of several types of cancer. Though the regulation of PRMT5 expression is poorly understood, and the full complement of its binding partners is yet unknown, it appears that in many cases the localization of the protein is relevant to its ability to transform cells.

The experimental work presented for the first time in this document serves to elucidate PRMT5’s phenotype and relevance in human melanoma. As suggested by the previous papers as well as the data presented here, PRMT5 represents a potentially useful marker of tumor staging, and may play diverse roles in individual melanoma subclasses – genetic, pathologic, or both. However, many questions still remain. The significance of upregulation of PRMT5 in early melanocytic nevi must first be clarified. Similarly, it will be necessary to identify the predictive markers of melanoma subsets which, following loss of PRMT5 protein, exhibit either increased or decreased proliferation. While the work presented here serves to enrich the understanding of PRMT5 in melanoma, other important questions must be addressed before PRMT5 may be considered a therapeutic target.
Study Rationale

The worldwide incidence of melanoma is rising faster than that of any other cancer. Although early stage disease can be successfully treated with surgery, advanced stage melanoma remains a devastating disease, with a five-year survival rate of approximately 15%. Few therapies exist for advanced melanoma, and available treatment regimens work for a limited time period. Thus there is a pressing need for a greater understanding of melanoma cell biology and new treatment strategies.

Post-translational modification of proteins is involved at all levels of cellular regulation. A family of enzymes called protein arginine methyltransferases (PRMT) functions primarily to catalyze the attachment of methyl (CH₃) groups to the guanidino nitrogen atoms of arginine amino acid residues. To date, eleven unique PRMT proteins have been identified in humans. These proteins are highly homologous and share a central catalytic domain (Figure 3). PRMT enzymes can methylate their targets in either a symmetric configuration (Type II PRMTs) or asymmetric configuration (Type I PRMTs). Enzymes in this family are generating increased interest as novel therapeutic targets, however their biologic role has not been fully characterized across a variety of tumor types.

The PRMT5 enzyme is a Type II arginine methyltransferase that can regulate various cellular functions including apoptosis, Golgi structure, pluripotency, cell growth and snRNP biosynthesis. Although prior studies have provided initial insight into mechanistic features of the PRMT enzymes, most data are derived from a limited panel of cell lines. It is likely that PRMT5 plays a unique role across individual tumor types. Indeed, PRMT5 over-expression has recently been shown to influence progression of leukemia, lymphoma, breast cancer and glioma. However,
there is currently only one publication describing the role of PRMT5 in melanoma. The recent report has demonstrated that in \textit{BRAF} wild type (but not mutant) melanoma cell lines, PRMT5 regulated ERK signal transduction amplitude in hepatocyte growth factor-stimulated cells\textsuperscript{93}. However, neither this publication nor any to date have demonstrated PRMT5 expression in primary melanoma specimens or its relationship with key transcription factors or cell cycle regulatory proteins in cells of the melanocytic lineage.

We hypothesized that PRMT5 plays a unique role in regulating the growth of human melanoma. In the present study, we demonstrate that PRMT5 expression is significantly elevated in melanocytic cells with dysregulated growth properties. In patient specimens, PRMT5 was upregulated in both the nucleus and the cytoplasm of melanocytic nevi, primary malignant melanoma, and metastatic melanoma, compared to normal epidermis. In human metastatic melanoma cell lines, PRMT5 was expressed predominantly in the cytoplasm, and found to be associated with its enzymatic cofactor Mep50. However, histologic examination of tumor xenografts in athymic mice revealed a heterogeneous pattern of nuclear and cytoplasmic PRMT5 expression. PRMT5 regulated melanoma cell growth, as siRNA-mediated depletion of PRMT5 inhibited proliferation in a subset of cell lines, but accelerated the growth of others. PRMT5 depletion also led to reduced expression of MITF (microphthalmia-associated transcription factor), a melanocyte-lineage specific oncogene, and increased the cell cycle protein p27\textsuperscript{kip1}. These results represent the first characterization of PRMT5 expression in clinical melanoma specimens and indicate this protein can regulate melanoma cell growth via pathways involving MITF and p27.
Chapter 2 - Materials and Methods

Cell lines and Reagents

The A375, Hs294T, MeWo and CHL-1 human melanoma cell lines were obtained from the ATCC (Manassas, VA). The 1106Mel and FO-1 human melanoma cell lines were a kind gift from Dr. Soldano Ferrone (University of Pittsburgh, Pittsburgh, PA). The WM1366 human melanoma cell line was obtained from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Human epidermal melanocytes (HEM) were purchased from ScienCell Inc. (San Diego, CA, USA). A375, Hs294T, and CHL-1 cells were cultured in DMEM (10% FBS, 1% antibiotics). MeWo cells were cultured in MEM (10% FBS, 1% antibiotics). 1106Mel, FO-1, and WM1366 cells were cultured in RPMI (10% FBS, 1% antibiotics). All cell lines were maintained at 37°C, 5% CO₂.

Antibodies against the following targets were used for immunohistochemistry (IHC), immunoblot (IB), or immunoprecipitation (IP): rabbit-anti PRMT5 (Abcam #ab31751; Cambridge, MA, USA), rabbit anti-PRMT5 (Cell Signaling #2252; Danvers, MA, USA), mouse-anti β-actin (Sigma #A5441-2; St. Louis, MO, USA); rabbit anti-GAPDH (Sigma #G8795); goat anti-lamin B (Santa Cruz #sc-6216; Santa Cruz, CA, USA), mouse anti-MITF (Thermo-Scientific #MS-772-PO; Lafayette, CO, USA), rabbit anti-MITF (Spring Bioscience #E17904; Pleasanton, CA, USA), mouse anti-cyclin D1 (Abcam #ab6152), rabbit anti-cyclin D1 (Cell Signaling #2978), rabbit anti-Mep50 (Bethyl Laboratories #A301561A; Montgomery, TX, USA), mouse anti-Mep50 (Abnova #79804; Taipei, Taiwan), rabbit anti-STAT3 (Cell Signaling #9132), rabbit anti-p27Kip1 (Cell
Signaling #2552), rabbit anti-p53 (Santa Cruz #sc-6243), mouse anti-p53 (Santa Cruz #sc-126), rabbit anti-PTEN (Cell Signaling #9559), rabbit anti-SYM10 (Millipore #07-412), anti-H4R3me2s (Abcam #ab5823).

FLLL32 and BLL-1, small molecule inhibitors of STAT3 and Type II PRMT enzymes (respectively), were designed and synthesized Dr. Chenglong Li and Dr. Pui-Kai Li, in the College of Pharmacy at The Ohio State University.

Melanoma patient specimens

All studies were approved by the Institutional Review Board of The Ohio State University (Protocol # 20100071; P.I. Lesinski; Protocol #2002H0089; co-P.I. Peters). Data on PRMT5 expression were obtained from a total of 21 normal epidermis (including both melanocytes and keratinocytes), 26 melanocytic nevi, 135 malignant melanoma, and 66 metastatic melanoma human tissue samples (Table 4). These formalin-fixed paraffin embedded specimens were obtained as commercially available tissue microarrays (Tissue Array Network Inc), arrays from the AIDS and Cancer Specimen Resource (University of California, San Francisco AIDS and Cancer Specimen Resource (ACSR) (http://acsr.ucsf.edu)), or individual de-identified specimens from The Ohio State University Medical Center.

Immunohistochemical analysis of PRMT5 expression

Slides were deparaffinized and rehydrated through graded alcohols and xylene. Antigen retrieval was performed in a solution of citrate buffer in a vegetable steamer. Rabbit polyclonal PRMT5 (Abcam #ab31751) was used at a dilution of 1:70. The secondary detection system used was Mach 4 Alkaline Phosphatase (Biocare Medical;
Concord, CA, USA) with Vulcan Fast Red chromogen (Biocare Medical). Slides were stained on a Dako Autostainer (Dako; Carpinteria, CA, USA), counterstained with hematoxylin, and permanently mounted. All samples were stained in duplicate, from serially-sectioned slides. Each specimen was evaluated under light microscopy and analyzed in a blinded manner under the direction of a board-certified dermatopathologist (S. Peters, M.D.). The percentage of PRMT5 positive cells in each sample was recorded, then sorted into one of four categories: negative (0-5% of cells in sample), low (6-25%), medium (26-75%), or high (>75%) percentage of PRMT5 positive cells. Each sample was further sub-categorized in terms of the percentage of cells positive for PRMT5 in either the nuclear or cytoplasmic compartment. Intensity of PRMT5 staining in both the nucleus and cytoplasm was qualitatively assessed. PRMT5 negative samples were recorded as zero; positive samples were scored from 1 to 5, 1 = low intensity and 5 = highest intensity.

Serial sections from all arrays and individual specimens were stained in duplicate for PRMT5. Of the original number of specimens available, 22 were discarded due to inadequate, lost, or damaged tissue, poor tissue quality, or discordant results between duplicates as determined by the dermatopathologist.

**Immunoblot analysis**

Total cell lysates from melanoma cell lines were subjected to immunoblot analysis. Briefly, equal cell numbers were lysed in equal volumes of total cell lysis buffer (5% SDS, 500mM Tris pH 6.8, 500µM EDTA; protease and phosphatase inhibitors, Thermo Scientific) for 30 min on ice. Then, each sample received an equivalent volume of Laemmli buffer (with 5% β-mercaptoethanol) and was boiled for 5 min at 100°C.
Proteins were resolved via SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies (Ab) to human PRMT5 (rabbit), MITF (rabbit), Mep50 (mouse), Cyclin D1 (mouse), STAT3 (rabbit), GAPDH (rabbit), lamin B (goat), SYM10 (rabbit), H4R3me2s (rabbit), p53 (rabbit/mouse), PTEN (rabbit), or β-actin (rabbit). Following incubation with the appropriate horseradish-peroxidase-conjugated secondary Ab, blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

**Subcellular fractionation**

Subcellular fractionation was performed using the NE-PER buffer kit (Thermo-Scientific #78833). Briefly, 5x10⁶ – 1x10⁷ cells were lysed according to manufacturer’s instructions, with the following modifications: 500-1000 µL of CERI and 11-55 µL of CERII lysis buffer were used. Cytoplasmic proteins were obtained by lysing cell pellets on ice for 20 min, followed by centrifugation at 20,000 x g for 10 min at 4°C. Supernatant was transferred to a clean tube. The remaining nuclear pellet was washed twice with cold PBS and centrifuged for 3 min at 16,000 x g at 4°C, then lysed in 100-200 µL of NER lysis buffer. Concentration of cytoplasmic and nuclear protein fractions was measured by Bradford assay. 8-10 µg were analyzed by SDS-PAGE, and validated using the nuclear and cytoplasmic markers lamin B and GAPDH, respectively.

**Orthotopic mouse tumor model**

All animal studies were conducted according to the guidelines of The Ohio State University Institutional Animal Care and Use Committee (IACUC) under an approved protocol (OSU protocol #2009A0178). Briefly, A375 or Hs294T human melanoma cells
(2x10^6 cells in 100 µL PBS) were injected subcutaneously into the flanks of 4-6 wk female athymic Balb/c^\text{nu/nu} mice (Harlan, Madison WI) as previously described. Tumors were harvested, fixed in 10% formalin, embedded in paraffin and sectioned at 4 µm for IHC analysis of PRMT5 protein.

**Immunoprecipitation**

5x10^6 – 1x10^7 cells were lysed in MPER lysis buffer (Thermo-Scientific #78501). 10% of cell lysate was saved as input control and an equivalent volume of Laemmli buffer was added. Lysates were pre-cleared for 30 min at 4°C on a rotating platform using True-Blot anti-rabbit IgG beads (eBiosciences #00-8800-25; San Diego, CA, USA). Samples were centrifuged at 10,000 x g for 3 min, and the supernatant was transferred to a clean tube and incubated with normal rabbit IgG, or rabbit primary antibody against Mep50, cyclin D1, or STAT3 for 1h at 4°C on a rotating platform. Samples were incubated with anti-rabbit IgG beads for 1h at 4°C, then centrifuged at 10,000 x g for 3 min at 4°C. Beads were washed three times in MPER lysis buffer, and protein complexes were retrieved by boiling samples at 100°C for 5 min in Laemmli/5% beta-mercaptoethanol buffer. Input, IgG control, and precipitated samples were then assessed via immunoblot.

**Depletion of PRMT5 via siRNA**

PRMT5 was depleted from melanoma cells using custom siRNA duplexes from Dharmaco (Thermo Scientific, Oligo ID# ATTAA-000838; Lafayette, CO, USA). siRNA oligonucleotides targeted the following sequence in exon 15 of the PRMT5 transcript: 5’-CCGCUAUUGCACCUGGAA-3’.  Silencer Select negative control siRNA (Ambion
Invitrogen, Carlsbad CA, USA) was used as control. Cells were seeded 24h in advance, then transfected using Lipofectamine 2000 (Invitrogen, Carlsbad CA) per manufacturer’s instructions. Transfection was performed using OptiMEM minimal serum media (Gibco #31985; Invitrogen, Carlsbad CA, USA) in 1.5 mL final volume. 6 hours following transfection, media was supplemented with 1.5 mL tissue culture media containing 20% FBS.

**Magnesium tetrazolium (MTT) proliferation assay**

The effects of siRNA-mediated PRMT5 depletion on cell proliferation were tested in a panel of human melanoma cell lines. Cell proliferation was measured as optical density (O.D.) at 570 nm using the MTT Cell Proliferation Assay Kit according to manufacturer’s instructions (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); ATCC, Manassas, VA, USA). Each assay was performed in assay replicates of five wells per condition.

**BLL-1, a small molecule inhibitor of Type II PRMT enzymes**

Molecular modeling was performed on the SAM binding region of PRMT5, and the small molecule BLL-1 was designed to bind and inhibit interaction with SAM. BLL-1 was resuspended in PBS. Melanoma cells were treated for 24h with PBS control or between 2-10 µM BLL-1. Floating and adherent cells were then harvested by decanting and trypsinization, respectively, and assessed for apoptosis via annexin V/propidium iodide staining and flow cytometry as described previously.\(^{113}\)
**Annexin V/Propidium Iodide Apoptosis assay**

A subset of cell lines was seeded 24h in advance and treated for 24h with increasing concentrations of vehicle control or BLL-1, a small molecule inhibitor of Type II PRMT enzymes. Adherent and floating cells were harvested by decanting or trypsinization, combined together within each condition, and 1x10^6 cells were stained with annexin V/propidium iodide (AV/PI) and assessed via flow cytometry for apoptosis. A minimum of 10,000 events were collected. Cells which were positive for both annexin V and propidium iodide were quantified as percentage of total gated cells. N=2 biological replicates per treatment condition were assessed.

**FLLL32, small molecule inhibitor of STAT3 phosphorylation and activation**

The Hs294T melanoma cell line was seeded 24h in advance and treated with DMSO control or increasing concentrations of FLLL32 for 6h and 16h in order to selectively inhibit phosphorylation and activation of STAT3 (as previously described)^56. A range of doses from 2-8 µM was used. Sublethal concentrations (2µM) were previously identified using annexin V/propidium iodide and PARP cleavage. Apoptosis of melanoma cells was induced at 6-8 µM at 16h.

**Statistical Analysis.**

For analysis of PRMT5 expression in patient specimens, Fisher’s exact test was used to compare the dichotomous outcome of PRMT5 > 5% vs. ≤ 5% (localized in either the nucleus or cytoplasm) by specimen type. The Wilcoxon rank sum test was used to determine if the percentage of positive cells differed by specimen type, examining the subcellular compartments separately.
Chapter 3 – Results

PRMT5 protein is upregulated in human malignant and metastatic melanoma tissues.

PRMT5 protein expression in human melanoma specimens was evaluated by IHC in 248 human tissues from individual patient samples and two separate tissue microarrays. Included in these samples were normal skin specimens, melanocytic nevi, primary malignant melanoma tumors (in situ and Clark’s stage I-IV), and metastatic melanoma tumors (Table 1). Initially, the overall percentage of PRMT5 positive (PRMT5+) cells in each specimen was assessed. Samples which contained 5% or fewer PRMT5+ cells were considered negative, while samples with greater than 5% PRMT5+ cells were recorded as positive. Overall, the number of samples with greater than 5% PRMT5+ cells was significantly higher in malignant (121 of 135 total samples were PRMT5+) and metastatic (58 of 66 total samples were PRMT5+) melanoma specimens as compared to cells of the normal epidermis (1 of 21 total samples were PRMT5+; p<0.0001, Fisher’s exact test) (Table 5 and Figure 5A). Many clinically relevant molecular changes in melanoma (e.g., $BRAF^{V600E}$ mutation, STAT3 Tyr$^{705}$ phosphorylation) have also been detected in melanocytic nevi, suggesting early events can trigger aberrant growth or transformation$^{114-116}$. Therefore, we also conducted an analysis of a small heterogeneous group of melanocytic nevi, and observed a significant increase in PRMT5+ specimens as compared to normal skin (23 of 26 melanocytic nevi were PRMT5+, compared to 1 of 21 samples from normal epidermis, p<0.0001, Fisher’s exact test).
The staining intensity of PRMT5 in human tissue specimens was also assessed. Due to heterogeneity of tissue quality and processing, and optimization of antibody concentration, data from each of two tissue arrays were analyzed independently. Overall, there was no difference in staining intensity within either the nucleus or cytoplasm between normal epidermis, melanocytic nevi, malignant, or metastatic tumor tissue. However, in both tissue arrays there appeared to be a positive correlation between PRMT5 staining intensity and the percent of cells positive in the specimens. This was true for both nuclear staining (Spearman correlation 0.79 (array 1) and 0.80 (array 2), \( p<0.0001 \)) and cytoplasmic staining (Spearman correlation 0.59 (array 1) and 0.81 (array 2); \( p<0.0001 \)) (data not shown).

**Heterogeneous localization of PRMT5 protein in patient melanoma specimens and human melanoma cell lines.**

Further examination of PRMT5 expression in human melanoma patient samples revealed that there was marked heterogeneity in subcellular localization of PRMT5 expression across patient samples (representative images in Figure 5B-C). For example, regardless of tumor stage, some individual patient melanoma specimens displayed predominantly nuclear staining (Figure 5B), while others displayed exclusively cytoplasmic staining (Figure 5C). Despite this heterogeneity, the percentage of cells positive for nuclear PRMT5 was significantly lower in metastatic tumors as compared to primary cutaneous malignant tumors (Table 6A; \( p\leq0.05 \), Wilcoxon rank sum). Additionally, malignant melanoma tissue displayed a significantly higher percentage of cytoplasmic PRMT5 positive cells as compared to normal epidermis (\( p<0.0001 \),
Wilcoxon rank sum), but a lower percentage of cytoplasmic PRMT5 positive cells when compared to melanocytic nevi (Table 6B; \( p \leq 0.05 \), Wilcoxon rank sum).

A series of studies was conducted in a panel of human metastatic melanoma cell lines to further explore the role of PRMT5 in melanoma cell biology. Similar to clinical samples, PRMT5 expression was detectable in cell lines derived from diverse organ sites and with unique genetic profiles, as well as normal human epidermal melanocytes (HEM) (Figure 6A). All cell lines expressed PRMT5 protein irrespective of \( BRAF \), \( NRAS \), or \( p53 \) mutational status. Since heterogeneity in PRMT5 localization was evident in many primary melanomas, subcellular fractionation was performed on each cell line to assess the presence of PRMT5 protein in cytoplasmic and nuclear compartments. The purity of fractions was confirmed via nuclear (Lamin b) and cytoplasmic (GAPDH) proteins. Immunoblot analysis revealed that PRMT5 protein was present in both the nuclear and cytoplasmic fractions of normal epidermal melanocytes, but was predominantly cytoplasmic in most human metastatic cell lines (Figure 6B). Of the 7 melanoma cell lines examined, only A375 cells expressed detectable levels of PRMT5 in the nucleus.

To examine the influence of tumor microenvironment on localization of PRMT5 \textit{in vivo}, A375 or Hs294T melanoma cells were implanted subcutaneously into the flank of athymic mice. Tumors were harvested, fixed, and IHC for PRMT5 was performed. Consistent with \textit{in vitro} analysis of these representative cell lines, PRMT5 protein was present in tumors from both cell lines. In both A375 and Hs294T tumors, PRMT5 expression was most abundant in the cytoplasmic compartment (Figure 6C). However, in specimens from both cell lines, between 35-65\% of cells were also positive for PRMT5.
in the nucleus. These data illustrate that interactions with the tumor microenvironment or three-dimensional tumor growth may impact the localization of this protein.

**PRMT5 associates with Mep50, but not cyclin D1 or STAT3, in melanoma cell lines**

Recent studies performed in other models have documented a physical and functional relationship between PRMT5, its enzymatic co-factor Mep50, the STAT3 transcription factor, and the cell cycle regulator cyclin D1. Based on the important role for these proteins in melanoma cell biology, it was of interest to determine whether these interactions also occurred in melanoma cells or if they were unique to those previously published cancer models. Therefore, we examined the expression level and association of these previously reported proteins with PRMT5. The PRMT5 cofactor Mep50 as well as cyclin D1 and STAT3, were expressed in all melanoma cell lines, and Mep50 was predominantly localized to the cytoplasm (Figure 6B and data not shown).

To assess the potential binding partners of PRMT5 in human melanoma cells, immunoprecipitation experiments were performed. Antibodies directed against human Mep50, cyclin D1, and STAT3 were used to precipitate these targets from whole cell lysates. PRMT5 was associated with Mep50, but not cyclin D1 or STAT3 in these cell lines (Figure 7A-B). Further experiments revealed that PRMT5 did not associate with cyclin D1 in either unsynchronized melanoma cells, or cells which underwent a double thymidine block and release, and were harvested in G1 and G2/M phases (data not shown).
PRMT5 does not bind to BRAF or CRAF in unstimulated melanoma cells

Based on data from a recent publication describing the role of PRMT5 in melanoma cell biology\textsuperscript{93}, we next examined whether PRMT5 physically associated with BRAF and/or CRAF in our panel of human melanoma cell lines. IPs against BRAF or CRAF were performed, and assessed via IB for B/CRAF as well as PRMT5 protein. BRAF and CRAF were successfully precipitated from the cell lysates; however, we observed no evidence of an association between PRMT5 and either BRAF or CRAF, in either BRAF WT or mutant cell lines (Figure 8). This observation was consistent for all cell lines examined regardless of the mutational status of BRAF.

siRNA-mediated depletion of PRMT5 modulates melanoma cell proliferation

To determine the effects of PRMT5 loss on melanoma cell function, we utilized siRNA oligonucleotides targeting exon 15 of PRMT5 mRNA\textsuperscript{66}. A panel of human melanoma cell lines was transfected with siRNA directed against PRMT5 or a scrambled negative control sequence. Cells were harvested at 24, 48, and 72h post transfection to assess PRMT5 expression and cell proliferation. siRNA decreased PRMT5 protein expression at 24, 48, and 72h, but did not induce apoptosis as measured by caspase-mediated cleavage of PARP (data not shown). Depletion of PRMT5 in the 1106Mel, FO-1, Hs294T, and WM1366 cell lines resulted in growth inhibition at 48h compared to scrambled siRNA control (between 11-28% growth inhibition, n≥3) and 72h (between 15-46% growth inhibition, n≥3; Figure 9). Interestingly, depletion of PRMT5 in the A375 and MeWo cell lines resulted in a robust increase in proliferation at 48h (29-37% increase in proliferation) and 72h (18-33% increase in proliferation; n≥3). In all
experiments, cells were harvested in parallel at 48h and 72h, to confirm depletion of the PRMT5 protein (Figure 9).

Depletion of PRMT5 modulates expression of the MITF transcription factor and \( p27^{\text{Kip1}} \)

We further delineated how PRMT5 loss alters key proteins that regulate melanoma cell proliferation. Of particular interest to our group were the effects of PRMT5 loss on expression of the MITF oncogene due to its established role in melanoma and the cyclin-dependent kinase inhibitor \( p27^{\text{Kip1}} \) which regulates cell proliferation. Depletion of PRMT5 resulted in the loss of MITF protein in 6 of 7 of melanoma cell lines, irrespective of BRAF or NRAS status. In 4 out of 6 cell lines which expressed \( p27^{\text{Kip1}} \), the expression of \( p27^{\text{Kip1}} \) increased upon depletion of PRMT5 (Figure 10).

PRMT5 does not physically associate with MITF, and MITF is not symmetrically dimethylated

In order to determine if PRMT5 physically associated with MITF, IP experiments targeting the MITF protein were performed on untreated 1106Mel, A375, and MeWo melanoma cell lines. This subset of cell lines was selected to represent both BRAF wild-type and mutant cells, and cell lines which increased or decreased proliferation in response to depletion of PRMT5 protein. MITF protein was efficiently precipitated from the 1106Mel and MeWo cell lines, but PRMT5 protein did not appear to co-precipitate with MITF (Figure 11A). In a similar manner, A375, 1106Mel, and MeWo cells were lysed and subjected to IP using the SYM10 antibody, which detects any proteins containing symmetrically dimethylated arginine residues. In Figure 11B, low molecular
weight proteins showing symmetric dimethylation were successfully immunoprecipitated from the samples. However, when the immunoprecipitated samples were subjected to MITF immunoblot, results revealed that there was no MITF protein present in the population of proteins with symmetrically dimethylated arginine residues. Thus, we concluded that MITF is not a methylation target of PRMT5 in unstimulated melanoma cell lines.

*siRNA-mediated depletion of PRMT5 results in altered protein level of PTEN but not p53*

PTEN (phosphatase and tensin homolog) is a protein which is frequently inactivated or lost in human melanoma. Therefore we examined the effect of PRMT5 depletion on PTEN protein level. The 1106Mel, MeWo, WM1366, and CHL-1 melanoma cell lines which were depleted of PRMT5 via siRNA and then assessed via IB for the tumor suppressor proteins PTEN and p53 (Figure 12). In three of the four cell lines, depletion of PRMT5 resulted in decreased PTEN protein.

Previous publications have described PRMT5’s role in regulating the transcriptional activity as well as the stability of p53 in response to DNA damage. In addition, p53 can also regulate checkpoint arrest in G1 of the cell cycle. Because PRMT5 physically interacts with p53 to stabilize it, and because we observed that depletion of PRMT5 has a significant effect on proliferation of melanoma cells, we next examined whether depletion of PRMT5 led to changes in p53 protein level. We observed that depletion of PRMT5 in these basal resting cells did not affect levels of p53 protein. It is important to note that these cells were not exposed to DNA damaging agents, and therefore the mechanism by which PRMT5 stabilizes p53 protein is not activated in resting cells. Interestingly, these cell lines represent a diverse range of p53
genotypes, both wild-type and various point and truncation mutants of p53. The doublet seen in the MeWo cell line following IB for p53 illustrates the unique p53 genotype, as MeWo cells are heterozygous for a mutation which encodes a premature stop codon\textsuperscript{117}.

*Treatment with FLLL32, a small molecule inhibitor of STAT3, decreases PRMT5 protein in melanoma cells*

Sequence analysis of the PRMT5 promoter suggests that STAT3, among other well known transcription factors, may regulate expression of PRMT5. Therefore, we used FLLL32, a small molecule inhibitor of STAT3, to examine whether inhibition of STAT3 activity modulated protein levels of PRMT5. Hs294T melanoma cells were seeded 24h in advance, and then treated with 2-8 µM of FLLL32 or DMSO vehicle control for 6h or 16h. Protein lysates were subjected to IB analysis of PRMT5, total STAT3, and \( \beta \)-actin loading control. FLLL32 appears to deplete PRMT5 protein at higher concentrations at both 6h and 16h (Figure 13). Previously published data of FLLL32 in melanoma confirm that phosphorylation of STAT3 decreases with this range of concentrations, at both 6h and 16h. It is also reported that at 16h, 1 µM of FLLL32 will result in PARP cleavage (a marker of apoptosis). However, in these experiments shown here, total amount of STAT3 protein was not affected. Further experiments using FLLL32 in the presence of pan-caspase inhibitors may provide additional information on the transcriptional regulation of PRMT5.
**BLL-1, a small molecule inhibitor of Type II PRMT enzymes, induces apoptosis in a panel of melanoma cell lines**

In order to test the effectiveness of a first-generation small molecule inhibitor of Type II PRMT enzymes in melanoma, cell lines were treated with BLL-1, a competitive inhibitor of the SAM binding domain. Cell lines were treated in duplicate for 24h with increasing concentrations of BLL-1, or vehicle control. At 24h, cells were harvested and assessed via flow cytometry and AV/PI staining for apoptosis. Though each cell line showed slight variations in sensitivity to the drug, the EC50 appeared to be around 50µM at 24h. There appeared to be no association between BLL-1 induced apoptosis and BRAF status, as both wild-type and mutant BRAF cell lines were tested and underwent apoptosis at similar concentrations of BLL-1 (Figure 14).

*Inhibition of methyltransferase activity via BLL-1 results in loss of MITF protein and decreased methylation of H4R3*

BLL-1 was used in the next experiments to test whether PRMT5’s methyltransferase activity was specifically necessary for regulation of MITF protein as well as for PRMT5’s cytoplasmic localization. The A375 and Hs294T melanoma cell lines were seeded 24h in advance and treated for 24h with a range of concentrations of BLL-1, or vehicle control. Subsequent protein analysis revealed that increasing concentrations of BLL-1 resulted in depletion of the MITF protein as well as nearly complete abrogation of symmetric methylation of H4 at arginine 3. The concentration required to effect these changes in A375 was lower than that of Hs294T, suggesting some difference in response among cell lines (Figure 15A).
Since the localization of PRMT5 appears to be biologically relevant, we sought to determine whether this localization is dependent on its methyltransferase activity. Cells treated for 24h with increasing concentrations of BLL-1 were harvested and subjected to subcellular fractionation. Subsequent IB analysis revealed that the fractions were largely pure, with the nuclear marker lamin B appearing only in nuclear fractions, and the cytoplasmic marker GAPDH (glyceraldehyde-3-phosphate dehydrogenase) appearing only in cytoplasmic fractions. BLL-1 treatment did not appear to affect the localization of PRMT5, which in the A375 cell line was almost exclusively cytoplasmic, with trace amounts in the nucleus (Figure 15B). However, it appeared that the highest concentration of BLL-1 treatment resulted in loss of MITF protein, which in lower concentrations appeared to concentrate exclusively in the nucleus.
Table 4. Distribution of human melanoma and normal skin samples obtained for assessment of PRMT5

<table>
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<th>Count</th>
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</tr>
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Table 5. The arginine methyltransferase PRMT5 is significantly overexpressed in human melanocytic nevi, malignant and metastatic tissues compared to normal epidermis.

<table>
<thead>
<tr>
<th>% of PRMT5 positive cells per sample:</th>
<th>&lt;5% (neg)</th>
<th>≥5% (pos)</th>
<th>Total</th>
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<td>* normal epidermis</td>
<td>20 (95%)</td>
<td>1 (5%)</td>
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<tr>
<td>melanocytic nevi</td>
<td>3 (12%)</td>
<td>23 (88%)</td>
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<tr>
<td>malignant</td>
<td>14 (10%)</td>
<td>121 (90%)</td>
<td>135</td>
</tr>
<tr>
<td>metastatic</td>
<td>8 (12%)</td>
<td>58 (88%)</td>
<td>66</td>
</tr>
</tbody>
</table>

* p<0.001 compared to melanocytic nevi, malignant, and metastatic tissue. Fisher's exact test
Table 6. The distribution of nuclear and cytoplasmic PRMT5 differs significantly among normal epidermis, melanocytic nevi, and malignant and metastatic tumors.

<table>
<thead>
<tr>
<th>A</th>
<th>% of cells per specimen which are nuclear positive:</th>
<th>number (and %) of patient specimens in each category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-5% (neg)</td>
</tr>
<tr>
<td>normal epidermis</td>
<td>20 (95%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>melanocytic nevi</td>
<td>1 (4%)</td>
<td>4 (7%)</td>
</tr>
<tr>
<td>malignant</td>
<td>24 (18%)</td>
<td>32 (24%)</td>
</tr>
<tr>
<td>metastatic</td>
<td>23 (35%)</td>
<td>19 (29%)</td>
</tr>
</tbody>
</table>

* p<0.001 in comparison with normal epidermis (*), melanocytic nevi (††), malignant (†), metastatic ($).

<table>
<thead>
<tr>
<th>B</th>
<th>% of cells per specimen which are cytoplasmic positive:</th>
<th>number (and %) of patient specimens in each category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-5% (neg)</td>
</tr>
<tr>
<td>normal epidermis</td>
<td>19 (95%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>melanocytic nevi</td>
<td>5 (20%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>malignant</td>
<td>34 (26%)</td>
<td>14 (11%)</td>
</tr>
<tr>
<td>metastatic</td>
<td>19 (32%)</td>
<td>2 (3%)</td>
</tr>
</tbody>
</table>

* p≤0.05 in comparison with normal epidermis (*), melanocytic nevi (††), malignant (†), metastatic ($).
Figure 5. PRMT5 is upregulated in human malignant melanoma tumors compared to normal epidermis, and subcellular localization of PRMT5 is heterogeneous amongst patient tumors. A, Brightfield image of a Stage 2/3 tumor and its adjacent normal epidermis, following IHC for PRMT5. PRMT5 protein is visualized with Vulcan FastRed label (pink); samples were counterstained with hematoxylin to delineate nuclei and connective tissue (blue). B, malignant skin tumor of pathologic stage 4, showing predominantly nuclear PRMT5 (arrows). C, malignant skin tumor of pathologic stage 4, showing predominantly cytoplasmic PRMT5 (arrows indicate hematoxylin-stained nuclei).
A. Total cell lysates from human epidermal melanocytes (HEM) or a panel of human metastatic melanoma cell lines were subjected to SDS-PAGE and immunoblot to demonstrate the presence of PRMT5 protein. β–actin was used as loading control. 

B. HEM and melanoma cell lines were subjected to subcellular fractionation and subsequently analyzed via immunoblot for PRMT5 and Mep50, as well as known markers for nuclear (lamin B) and cytoplasmic (GAPDH) fractions. 

C. 1x10^6 cells from the A375 and Hs294T human melanoma cell lines were implanted into the flank of immunocompromised mice and allowed to grow for approximately 21 days. Tumors were removed, fixed in formalin, and analyzed via IHC for PRMT5. Data represent total percentage of cells in each tumor which were positive for PRMT5 in nucleus, cytoplasm, or both. n≥2 tumors were analyzed.

Figure 6. PRMT5 is present in and displays heterogeneous localization between human cell lines and orthotopic tumors.
A. Unsynchronized HEM as well as a subset of melanoma cell lines were harvested and subject to total cell lysis followed by immunoprecipitation. 10% of input protein, as well as protein from IgG control and IP directed against Mep50 and cyclin D1, was subjected to SDS-PAGE and immunoblotted for IP target proteins as well as PRMT5. B, IP against STAT3 was performed on the same cell line panel, and subject to immunoblot against STAT3 as well as PRMT5.

Figure 7. PRMT5 associates with Mep50, but not cyclin D1 or STAT3, in a subset of melanoma cell lines.

A, Unsynchronized HEM as well as a subset of melanoma cell lines were harvested and subject to total cell lysis followed by immunoprecipitation. 10% of input protein, as well as protein from IgG control and IP directed against Mep50 and cyclin D1, was subjected to SDS-PAGE and immunoblotted for IP target proteins as well as PRMT5. B, IP against STAT3 was performed on the same cell line panel, and subject to immunoblot against STAT3 as well as PRMT5.
A subset of melanoma cell lines were harvested and subject to total cell lysis followed by immuno precipitation. 10% of input protein, as well as protein from IgG control and IP directed against BRAF and CRAF, was subjected to SDS-PAGE and immunoblotted for IP target proteins as well as PRMT5.

Figure 8. PRMT5 does not bind BRAF or CRAF in unstimulated WT or mutant melanoma cells.

A subset of melanoma cell lines were harvested and subject to total cell lysis followed by immunoprecipitation. 10% of input protein, as well as protein from IgG control and IP directed against BRAF and CRAF, was subjected to SDS-PAGE and immunoblotted for IP target proteins as well as PRMT5.
A panel of melanoma cell lines was transiently transfected with scrambled siRNA or siRNA against PRMT5 and subjected to MTT proliferation assays at 48h and 72h post-transfection. Data represent percent of growth inhibition ± SEM (n≥3). Representative immunoblot shows PRMT5 and β-actin protein in control (-) and PRMT5 depleted (+) cells at 48h.

Figure 9. Depletion of PRMT5 via siRNA results in changes in proliferation. A panel of melanoma cell lines was transiently transfected with scrambled siRNA or siRNA against PRMT5 and subjected to MTT proliferation assays at 48h and 72h post-transfection. Data represent percent of growth inhibition ± SEM (n≥3). Representative immunoblot shows PRMT5 and β-actin protein in control (-) and PRMT5 depleted (+) cells at 48h.
A panel of melanoma cell lines was transiently transfected with scrambled siRNA or siRNA against PRMT5 and harvested at 48h post transfection. Total cell lysates were subjected to SDS-PAGE and immunoblot directed against PRMT5, MITF, p27\textsuperscript{KIP1}, and β-actin proteins. \(n\geq2\) transfections were performed; representative immunoblots are shown here.

Figure 10. Depletion of PRMT5 via siRNA results in decrease in MITF protein and increased p27\textsuperscript{KIP1}.
Figure 11. PRMT5 protein does not associate with MITF protein.  
A, IP against the MITF protein or IgG control was performed in 1106Mel and MeWo melanoma cell lines, and subject to immunoblot against MITF and PRMT5.  B, IP against symmetrically dimethylated proteins (SYM10 antibody) was performed in A375, 1106Mel, and MeWo melanoma cell lines.  LMW, low molecular weight region of SDS-PAGE gel; numbers represent protein size in kilodaltons (kDa).  Immunoblot was performed for SYM10 (symmetric arginine dimethylation) and MITF.
A subset of melanoma protein samples from previous siRNA experiments were further analyzed via SDS-PAGE for PTEN, p53, and β-actin protein. PTEN, p53 and β-actin were assessed at 48h post transfection, in a subset of unstimulated melanoma cell lines.

Figure 12. Depletion of PRMT5 modulates PTEN protein, but does not modulate p53 protein levels in a subset of cell lines.
Unsynchronized, unstimulated Hs294T melanoma cells were treated with increasing concentrations of FLLL32, harvested at 6h or 16h, and analyzed via SDS-PAGE and immunoblotting for PRMT5, total STAT3, and β-actin.

Figure 13. FLLL32, a small molecule inhibitor of STAT3, inhibits expression of PRMT5 protein in Hs294T melanoma cells. Unsynchronized, unstimulated Hs294T melanoma cells were treated with increasing concentrations of FLLL32, harvested at 6h or 16h, and analyzed via SDS-PAGE and immunoblotting for PRMT5, total STAT3, and β-actin.
A subset of melanoma cell lines were treated for 24h with increasing concentrations of BLL-1, a small molecule inhibitor of Type II PRMTs. Floating and adherent cells were harvested at 24h and assessed for apoptosis via annexin V/propidium iodide staining and flow cytometry.

Figure 14. BLL-1, a small molecule inhibitor of Type II PRMTs, induces apoptosis in melanoma cell lines.
Figure 15. The effect of BLL1, a PRMT Type II inhibitor, in melanoma.
A, BLL1 causes loss of MITF protein and loss of H4R3 methylation at low concentrations in a subset of melanoma cell lines. A subset of melanoma cells were treated with increasing concentrations of BLL-1 for 24h. Adherent cells were harvested for protein and immunoblots for MITF, symmetric dimethylation of H4R3 (H4R3me2s), and β-actin were performed. B, BLL-1 does not modulate subcellular localization of PRMT5 or MITF, but causes loss of MITF from the nucleus in A375 melanoma cells. In a preliminary experiment, A375 melanoma cells were treated for 24h with increasing concentrations of BLL-1. Adherent cells were harvested and subcellular fractionation was performed. 8-10 µg of nuclear or cytoplasmic proteins were subject to immunoblot for PRMT5, MITF, lamin B, and GAPDH.
Figure 16. Potential mechanisms which highlight the role of PRMT5 in melanoma cells.
Chapter 4 – Discussion

While our understanding of the role of PRMT5 in human biology is still in its infancy, the examination of this protein in human malignancies suggests that it is an effector of tumorigenesis. It appears to behave as an oncoprotein, a fact which may at the very least make PRMT5 a useful diagnostic marker, and potentially, a novel molecular therapeutic target. However, PRMT5’s activities and binding partners, and the circumstances under which these partners come together, remain poorly understood. This provides us with an important opportunity to further characterize its function so PRMT5 can be assessed as a potential therapeutic target in future studies.

In this report, we demonstrate for the first time that PRMT5 is upregulated in primary malignant and metastatic melanoma, as well as in melanocytic proliferations, taken from human patients. Using a panel of human metastatic melanoma cell lines, we further demonstrate that siRNA-mediated loss of PRMT5 led to reduced expression of MITF, increased expression of p27Kip1 and variable effects on cell proliferation. These data indicate a unique role for PRMT5 in regulating melanoma cell biology and proliferation.

In metastatic melanoma, poor survival rate coupled with limited success of the current treatment options highlights the need for a greater understanding of melanoma cell biology so that alternative treatment strategies can be identified. Two areas of research under exploration are the control of epigenetic modifications (such as acetylation, ubiquitination, and methylation) as well as discovering novel molecular
targets which may alter melanoma biology and significantly enhance the efficiency of existing chemotherapeutic treatments. We have focused our attention on a family of protein arginine methyltransferase (PRMT) enzymes.

Proteins reported to be targeted directly by the PRMT family are numerous, and include nucleosomal histones which mediate gene expression, as well as proteins such as p53, TRAIL, cyclin D1, STAT1, STAT3, and nascent cytoplasmic histones – each of which are involved in major regulatory processes such as proliferation, apoptosis, gene expression, and tumor suppression\textsuperscript{66-68,82,84,86-87,89,92-93,108,110}. Furthermore, over-expression of the PRMT5 family member has been shown to occur in the setting of advanced stage glioblastoma, lung cancer, leukemia and lymphoma\textsuperscript{74,87,111,118-119}. However, few if any of the interactions between PRMT5 and other proteins have been validated in the setting of melanoma. Data from the present study indicate that although PRMT5 is expressed at high levels in melanoma, the previously reported protein-protein interactions may differ depending upon the type of malignancy.

In the human patient samples assessed by IHC, PRMT5 protein was significantly increased in both the cytoplasmic and nuclear compartments of malignant and metastatic tumors, as well as in melanocytic nevi. This suggests that PRMT5 expression may fluctuate during melanoma progression, and the upregulation of PRMT5 may be an early event in tumorigenesis. The role for PRMT5 expression in melanocytic nevi also deserves further investigation, as these early proliferations display many molecular alterations, including BRAF mutations, which drive the development of malignant melanoma\textsuperscript{116}. Similarly, elevated PRMT5 may be a hallmark of increased proliferation in nevi or other stages of tumorigenesis. While not assessed in this study, future experiments could include a comparison between the level of PRMT5 protein
expression and the number of mitotic cells present in the nevus or tumor. Due to the limited clinical information available on the patient specimens, as well as the retrospective nature of this study, we could not yet determine if there is a statistically significant relationship between level of PRMT5 and tumor stage (Stage I-IV) among primary malignant tumors. Similarly, due to lack of clinical information we could not exclude a role for ultraviolet light exposure or previous clinical therapies as potential modifiers of PRMT5 expression in any of the patient samples which were assessed.

The pattern of subcellular localization of this protein was strikingly different among many patient samples. In Figure 5B and 5C, Stage IV primary skin tumors from two different patients exhibit different patterns of PRMT5 localization. Further characterization revealed that, despite individual heterogeneity, the percentage of cells positive for PRMT5 in the nucleus was significantly higher in melanocytic nevi compared to primary malignant tumors, and also significantly higher in primary malignant tumors compared to metastatic tissue (Table 6A). These observations suggest that the localization of PRMT5 may be a dynamic process which either promotes or occurs as a result of the progression of melanoma. To further understand differences in localization, we extended our observations of this protein in vitro using normal melanocytes and immortalized metastatic melanoma cell lines. PRMT5 was expressed in all cell lines surveyed (Figure 6A). In normal melanocytes, PRMT5 was detected in both the cytoplasmic and nuclear compartments. However, in cell lines derived from metastatic tumors, PRMT5 was largely restricted to the cytoplasmic compartment. These fractionation data were consistent with the observation that PRMT5 was predominantly cytoplasmic in metastatic tumor specimens. Although localization of PRMT5 in human patient specimens was more heterogeneous, it is also important to note that patient
tumors represent tissue exposed to various factors within the microenvironment including the milieu of cytokines, integrin interactions and variable regions of hypoxia. In contrast, in vitro studies cannot always account for complex interactions with surrounding stromal tissues, immune cells or differences in oxygen, as demonstrated in PRMT5 staining of orthotopic tumors derived from two melanoma cell lines (Figure 6C). The influence of these factors on localization of PRMT5 warrants further investigation.

As shown in Figure 6A, PRMT5 was demonstrated to be present in whole cell extracts of both normal human epidermal melanocytes and metastatic melanoma cell lines. Cytoplasmic and nuclear protein fractions were obtained from this panel of cell lines, and probed for PRMT5 and its co-factor, Mep50 (Figure 6B). Interestingly, in metastatic melanoma cell lines (which demonstrated largely cytoplasmic PRMT5), there was a separate pool of Mep50 in the nucleus of the cells which did not appear to be associated with PRMT5. Conversely, in normal HEMs, there appeared to be a separate pool of PRMT5 in the nucleus of the cells which did not appear to be associated with Mep50. It is conceivable that this difference may partially account for the basic differences in biology of these cells. It is important to further characterize the phenotypic effect of the loss of PRMT5 in each of these cell lines.

A previous report characterizing PRMT5 protein in human testicular cancer noted very pronounced differences in subcellular localization between normal testicular tissue and testicular tumors. This suggests its location in the cell has profound consequences with regard to the development of testicular cancer. Interestingly, and somewhat similar to what is seen in normal melanocytes and melanoma, PRMT5 is largely nuclear in normal testicular tissue, but translocates to the cytoplasm in testicular tumors. This suggests that PRMT5’s role in the nucleus, including gene expression, may be highly
relevant to incidence or progression of testicular cancer. Therefore, it is not inconceivable that the localization of PRMT5 would be different in melanoma compared to leukemia and lymphoma, and that this difference in localization would translate to a unique role in melanoma compared to myelo- and lymphoproliferative cancers. Dynamic changes in localization of PRMT5 appear to be relevant to normal fetal development and maintenance of stem cell pluripotency in mice, which also supports the hypothesis that PRMT5’s localization is a factor in melanoma biology.89

The absence of PRMT5 from the nucleus and its presence in the cytoplasm suggests that its role is not exclusive to control of gene expression in melanoma. Based on this expression pattern, it is likely to influence melanoma cell biology through interaction with relevant cytoplasmic factors. Previous reports in leukemia, lymphoma, and mouse embryonic stem cells have suggested several proteins with which PRMT5 can associate. To examine these relationships, we performed immunoprecipitation experiments in both normal melanocytes and a panel of metastatic human melanoma cell lines. Mep50, cyclin D1, and STAT3 were all present in the cytoplasm with the opportunity to bind PRMT5. However, PRMT5 was shown to co-precipitate with Mep50, but not with cyclin D1 or STAT3 (Figure 7A-B). Further experiments in synchronized cells confirmed that although cyclin D1 protein was expressed in all phases of the cell cycle, it did not associate with PRMT5 at any phase of the cell cycle. It is important here to contrast these studies with the previously published reports. Andreu-Perez et al. demonstrate a robust association between PRMT5 and a mutant form of cyclin D1 (T286A) in mouse lymphoma, but PRMT5 associated very poorly with wild-type cyclin D193. In 2010, Tee et al. showed an association with PRMT5 and STAT3 in wild-type mouse embryonic stem cells89. While increased STAT3 activity is associated
with melanoma, this association had not yet been examined in melanoma. In order to explore the possibility that interactions between PRMT5 and its targets are very transient, a crosslinking reagent might be used in the future to preserve this fragile interaction during IP. These preliminary experiments, demonstrating a lack of interaction between PRMT5 and cyclin D1 or STAT3 as well as differences in subcellular localization, suggest that the role of PRMT5 in melanoma may differ from its role in other cell types and cancers. No consistent relationship between biologic response of melanoma cells following PRMT5 siRNA and genotype was observed. However, further studies with a more extensive cell line panel and with a less strict immunoprecipitation protocol may be warranted, to determine if any correlation exists between genotype and response to PRMT5 depletion.

To date, only one other report has examined the role of PRMT5 in melanoma. In a study by Andreu-Perez et al., PRMT5 was shown to bind CRAF in PC12 and Cos-7 cells\textsuperscript{93}. Additionally, PRMT5 was shown to positively regulate activation of the ERK pathway in melanoma cells, in response to growth factor stimulation\textsuperscript{93}. We also conducted a number of exploratory studies to examine interactions between PRMT5, BRAF, and CRAF in a panel of unstimulated, \textit{BRAF} wild-type and \textit{BRAF} mutant melanoma cell lines. Our experiments show that PRMT5 did not associate with BRAF or CRAF in unstimulated, \textit{BRAF} wild-type melanoma cell lines. Our data also indicated a lack of association between PRMT5 and CRAF in \textit{BRAF}\textsuperscript{V600E} mutant cell lines. Further, we observed that CRAF was not symmetrically dimethylated in unstimulated melanoma cells regardless of genotype (data not shown). Several important differences exist between these experiments and the previously published report. Firstly, PRMT5 was not shown to interact with CRAF in melanoma cells, and secondly, that PRMT5 appears to
modulate the ERK pathway only in BRAF wild type cells (that have been activated with human growth hormone). Possibly due to CRAF transactivation, recently approved BRAF inhibitor therapies are not appropriate for use in BRAF wild-type tumors\textsuperscript{49} (Table 2). BRAF mutant tumors, though initially responsive to the Vemurafenib, eventually become resistant and tumors return\textsuperscript{120}. Because of this, it may be of interest in future studies to investigate whether or not PRMT5 represents a therapeutic target in BRAF wild-type tumors, and whether it has a role in regulating resistance to the BRAF inhibitor. Together the present study and that of Andreu-Perez et al. indicate that PRMT5 may play diverse roles in the context of BRAF status, in melanoma cell biology.

While it is important to understand the protein interactions between PRMT5 and other cellular components, we ultimately wanted to test what direct effect PRMT5 had on global cellular processes such as proliferation and apoptosis. Therefore, we turned to siRNA depletion and examined our panel of melanoma cell lines for apoptosis and proliferation. In each cell line, loss of PRMT5 failed to induce apoptosis (data not shown) but had a differential yet significant effect on proliferation. In the majority of cell lines, proliferation was inhibited. However, a subset of cell lines experienced an acceleration of growth. Importantly, this difference (seen in the A375 and MeWo cell lines) could not be attributed to BRAF status, as would most commonly be considered when studying melanoma. Though the genotypic data for some cell lines is incomplete, each cell line in the panel displays a unique genotype in some of the major melanoma cancer genes (Ras, RAF, p53, PTEN, p16). In addition, the diverse mutations seen in p53 in this panel of cell lines are not yet characterized as activating, inactivating, or alternatively functioning mutations. Therefore, it will be essential in future studies to examine more cell lines which have been genotyped for each of the aforementioned...
targets, in order to examine correlation between genotype and PRMT5 expression, localization, or role in proliferation. It is not yet clear at which phase of the cell cycle the cells arrest, following PRMT5 depletion. Some impediments to this analysis include the aneuploidy of most if not all of the cell lines; the relatively small changes in percent proliferation or growth inhibition (only between 15-40% change in proliferation may not be readily detectable by flow cytometry); similarly, the loss of PRMT5 may only alter the behavior of cells which are in a certain phase of the cell cycle, and would not be detected in these experiments which were performed on unsynchronized cells. Though the initial studies shown here have yielded concrete and intriguing results, these are a few of the technical challenges in performing transient siRNA experiments.

To understand how loss of PRMT5 regulated melanoma cell proliferation, we initially focused our attention to the MITF transcription factor. Previous studies have characterized MITF as a master regulator of normal melanocyte differentiation, as well as melanoma tumor and stem cell biology\textsuperscript{32,121-122}. In the present study, PRMT5 depletion was associated with decreased MITF protein in 6 out of 7 cell lines. Similarly, MITF protein was depleted when melanoma cells were treated with a small molecule inhibitor of the Type II PRMT enzymes (Figure 15A). Thus, several lines of evidence point to the involvement of PRMT5 in MITF protein expression. However, the mechanism behind this observation is unclear and likely quite complex. PRMT5 is most frequently associated with transcriptional repression, and displays cytoplasmic localization in most melanoma cell lines. Therefore it is less likely that PRMT5 interacts directly with the MITF promoter, but could possibly negatively regulate a protein which in turn represses MITF. Lastly, the effect of PRMT5 siRNA on the abundance of MITF
mRNA was heterogeneous and inconclusive, and within some cell lines did not correlate with changes in MITF protein seen in immunoblots (data not shown).

Consistent with reduced MITF protein levels, we observed an increase in the expression of p27\(^{\text{Kip1}}\), a cyclin-dependent kinase inhibitor (CKI) which regulates progression through the cell cycle. Interestingly, MITF has been implicated in regulating the stability of the p27\(^{\text{Kip1}}\) protein through an upstream effect on the expression of Dia1\(^{\text{123}}\). The Dia1 protein interacts directly with p27\(^{\text{Kip1}}\) and targets it for degradation via the ubiquitin-proteasome pathway. When MITF protein is depleted, expression of Dia1 decreases and p27\(^{\text{Kip1}}\) is protected from degradation\(^{\text{123-125}}\). This relationship is currently under investigation and may potentially explain the effect of loss of PRMT5 (and subsequent loss of MITF) on altered proliferation in melanoma (Figure 16). Since p27\(^{\text{Kip1}}\) is regulated by post-translational modification and proteasomal degradation, we have not yet ruled out the possibility that PRMT5 physically associates with p27\(^{\text{Kip1}}\) and affects its stability (Figure 16). Future studies examining the associations and post-translational modifications of p27\(^{\text{Kip1}}\), or the effect of a small molecule proteasome inhibitor in the context of MITF overexpression, might elucidate this pathway in melanoma cells. Though most cell lines demonstrate elevated levels of p27\(^{\text{Kip1}}\) and a concurrent decrease in proliferation, care must be taken before conclusions are drawn. In the A375 cells, there is an increase in p27\(^{\text{Kip1}}\), yet the decrease in MITF is modest at best (Figure 10), and this cell line (along with MeWo) exhibits growth acceleration (Figure 9). Thus, as previously stated, a larger panel of genotypically well characterized cell lines should be assessed in future experiments.

As mentioned previously, it is important to note that MITF expression is regulated through several complex, indirect mechanisms including stability via post-translational
modifications or the activity of the MEK/ERK pathway\textsuperscript{126}. It is known that MITF protein is regulated at multiple levels, including post-translational modification\textsuperscript{126-127}. Since MITF protein itself is routinely degraded via the ubiquitin-proteasome pathway\textsuperscript{126}, we next proposed that PRMT5 could directly modify this protein and regulate its stability. Immunoprecipitation experiments with an anti-MITF antibody and an antibody recognizing symmetrically dimethylated proteins (SYM10) further indicated that this protein did not associate with PRMT5, was not symmetrically dimethylated (Figure 11A-B), and therefore unlikely a target of PRMT5 methyltransferase activity. However, to more fully test this interaction, ongoing studies are assessing the effect of proteasome inhibitors such as MG132 or bortezomib on post-translational regulation of MITF protein\textsuperscript{128-129}. Ultimately, it appears that MITF protein persists in the cell in a balanced state, between stable expression of mRNA via MEK/ERK, and stable regulation of MITF protein degradation. As recent studies have begun to suggest that PRMT5 interacts with proteins in major signaling pathways (such as Rb/E2F\textsuperscript{130}), PRMT5 may impact either or both of these mechanisms (Figure 16).

Two other proteins, PTEN and p53, are highly relevant in both melanoma and other types of cancer. PTEN is most often mutated or lost in melanoma tumors, and alterations in the p53 gene or in the protein’s activity are seen in many solid cancers including lung and colon. In addition, p53 is a master regulator of CKI proteins, management of DNA repair, and subsequently the progression of cells through the cell cycle. Therefore, we chose to examine both of these proteins in PRMT5-depleted melanoma cells. PTEN appeared to increase in expression in a subset of cells, when PRMT5 was depleted. However, it is important to note that the PTEN status of each of these cell lines differs, when the status is indeed known. Therefore, the effect of
upregulation of PTEN protein (as well as p53) must be interpreted with caution. We observed that the levels of p53 protein did not change at all following depletion of PRMT5. This result should be taken in context of a previous report showing that PRMT5 positively regulates expression of p53 in osteosarcoma cells. In this report from 2008, PRMT5 bound to and methylated p53 under conditions of DNA damage, but these two proteins did not interact in basal conditions where DNA damage was not inflicted. It is possible that if we were to examine the expression of p53 plus or minus a DNA damaging agent, in the presence of PRMT5 or in PRMT5 depleted cells, that we may see an effect of PRMT5 on expression of p53. It will be important to understand the effect of PRMT5 depletion or inhibition in the context of DNA damaging agents, as this may support a combinatorial therapeutic approach using PRMT5 inhibitors and classical chemotherapies together to treat cancer.

The predominant transcription factor which regulates PRMT5 expression is not yet known. However, sequence analysis of the promoter region revealed, among others, a STAT3 (signal transducer and activator of transcription) consensus sequence. Therefore, we utilized FLLL-32, a small molecule inhibitor of STAT3, to examine the effect of STAT3 inhibition on expression of PRMT5. Subsequent experiments also involved the use of BLL-1, a small molecule inhibitor of Type II PRMTs. FLLL-32 and BLL-1 were both designed and synthesized by our collaborators in the College of Pharmacy at The Ohio State University. These experiments serve to add to the basic understanding of PRMT5 function, while at the same time examine whether PRMT5 could be an appropriate clinical target.

FLLL32, a previously described selective inhibitor of the STAT3 transcription factor, abrogates phosphorylation of STAT3 and subsequently downregulates genes
normally targeted by phospho-STAT3 dimers\textsuperscript{58-59}. A sequence analysis of the PRMT5 promoter revealed that among numerous potential transcription factors, STAT3 may recognize a consensus sequence in the promoter and regulate expression of PRMT5 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PRMT5). In Hs294T melanoma cells treated with increasing concentrations of FLLL32, it appears that PRMT5 protein decreased at higher concentrations (6-8 µM) at both 6h and 16h after single treatment. While this result is encouraging, it is important to note that the previous reports of FLLL32 in melanoma demonstrate that apoptosis is initiated by FLLL32 at much lower concentrations (1 µM) at 16h. Therefore, it could be possible that PRMT5 is depleted not due to transcriptional downregulation, but through effector caspase activity\textsuperscript{131}. Future experiments which measure PRMT5 mRNA expression in the presence of FLLL32, as well as those which measure FLLL32-mediated PRMT5 protein depletion in the presence of the pan-caspase inhibitor ZVAD-FMK\textsuperscript{132} will be essential.

Ultimately, the design of a small-molecule inhibitor which targets PRMT5 may serve as a potential approach for therapy of specific subtypes of malignant and metastatic melanoma or other tumors. BLL-1 is a small molecule lead compound which inhibits Type II PRMTs. This molecule is a direct inhibitor of the catalytic domain of the PRMT5 protein, preventing the docking of the methyl donor s-adenosyl-methionine\textsuperscript{133}. Preliminary studies in our laboratory have shown that treatment of a subset of melanoma cells with increasing concentrations of BLL-1 over 24h resulted in apoptotic cell death in a concentration-dependent manner (Figure 14). While there was some variation in response, BLL-1 appeared to induce apoptosis in both \textit{BRAF} wild-type and mutant cell lines at the same EC50 concentration (50 µM). It is important to note here that BLL-1 resulted in melanoma cell apoptosis, while depletion of PRMT5 specifically via siRNA did
not induce apoptosis (data not shown). Therefore, these data suggest a number of possible scenarios: 1) BLL-1 targets more than one Type II PRMT besides PRMT5, and 2) BLL-1 may have previously unappreciated off-target effects on other proteins not associated with the PRMT family. The results of both the BLL-1 studies and the siRNA studies also suggest that the Type II PRMTs may serve some redundant function, where for example PRMT7 may compensate for PRMT5’s activities if PRMT5 is mutated or lost.

Since the previously described experiments suggest that PRMT5 is a major regulator of MITF expression, we examined the level of MITF protein in A375 and Hs294T melanoma cells treated with increasing concentrations of BLL-1 for 24h. Immunoblotting for MITF, H4R3me2s, and β-actin were performed. In cells treated with higher concentrations of BLL-1, MITF protein as well as methylation of H4R3 decreased (Figure 15A). These data further substantiate our observations that PRMT5 at least partially regulates the MITF protein. Interestingly, BLL-1 affected the methylation status of histone H4, which is an event that is typically thought to occur in the nucleus of the cell. This result is intriguing, as we previously showed that PRMT5 exists exclusively in the cytoplasm of Hs294T cells, and therefore would not have access to nucleosomal histone H4. This result does not rule out the possibility that another Type II PRMT can perform the same methylation modification, that BLL-1 in fact targets more than one Type II PRMT or an entirely different protein, or quite possibly that methylation of histones can occur in the cytoplasm prior to their association with chromatin (Figure 16). It is also important to consider that, just as in treatment with the STAT3 inhibitor FLLL32, these concentrations of BLL-1 are in the range which induces apoptosis. Further studies with lower concentrations of both FLLL32 and BLL-1, in the
absence or presence of caspase inhibitors, may also be an informative means to assess the mechanism of PRMT5 regulation and activity.

Treatment with BLL-1 also allowed us to investigate whether or not the subcellular localization of PRMT5 was dependent on its methyltransferase activity. In Figure 15B, we show that in subcellular fractions of A375 melanoma cells treated with increasing concentrations of BLL-1, PRMT5 is found almost exclusively in the cytoplasm, with trace amounts in the nucleus, and that treatment with BLL-1 does not alter the localization of PRMT5. Conversely, the MITF protein is found exclusively in the nucleus but is completely absent in both the nucleus and the cytoplasm in cells treated with 100 

µM BLL-1. GAPDH, lamin B, and PRMT5 proteins are still abundant in cells treated with this concentration, which suggests but does not prove that caspase-mediated protein degradation does not explain the loss of MITF protein. However, as suggested previously, it may be useful to examine levels of MITF protein in cells treated with sub-lethal concentrations of BLL-1 in the absence or presence of a caspase or proteasome inhibitor.

Each of these preliminary results support the hypothesis that PRMT5 plays a role in human melanoma, and may represent a useful clinical target in at least a subset of patient tumors. Although inhibition of this protein can induce growth arrest in many situations, loss of PRMT5 was not cytotoxic. Therefore, future studies will assess whether PRMT5 inhibition in combination with other existing chemotherapies or immunotherapies may be a promising approach for future studies. The variable biologic response to PRMT5 loss in human melanoma cell lines underscores the importance of future mechanistic studies, in order to understand PRMT5’s mechanism of action, and its suitability as a molecular target in human melanoma cells.
In conclusion, we demonstrate that PRMT5 is expressed in human melanoma and melanocytic nevi. Although it was expressed predominantly in the cytoplasm of human melanoma cell lines, clinical and orthotopic tumor data indicate that cellular localization of PRMT5 may be modulated by the tumor microenvironment. PRMT5 also appears to play a unique role in regulation of key factors including MITF, \( p27^{Kip1} \), and PTEN, in order to mediate melanoma cell growth, while the interactions of PRMT5 with other proteins may be distinctly different in human melanoma cells. Together these results highlight important factors that deserve consideration, as therapeutic strategies move toward targeting PRMT5 or other methyltransferases in melanoma.
Bibliography


