Decoding PRMT1: Studies On the Catalytic Mechanism, Regulation, Inhibition, and Crosstalk of PRMT1-Dependent Methylation

Heather L. Rust
University of South Carolina

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DECODING PRMT1: STUDIES ON THE CATALYTIC MECHANISM, REGULATION, INHIBITION, AND CROSSTALK OF PRMT1-DEPENDENT METHYLATION

by

Heather Lynn Rust

Bachelor of Science
Saint Francis University, 2008

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Accepted by:
Paul Thompson, Major Professor
Caryn Outten, Committee Member
Sheryl Wiskur, Committee Member
Lorne Hofseth, Committee Member
Lacy Ford, Vice Provost and Dean of Graduate Studies
Dedication

I dedicate this dissertation to my Mom, Dad, and brother Ryan for the unconditional love and support that they have given me throughout my entire life. Mom, I am so proud to be your daughter and so grateful that I can call you best friend. You have always been there for me and believed in me no matter what and I couldn’t have made it this far without you. Dad, you have always put our family first and therefore have given me the chance to have a wonderful life, and for that I will always be grateful. You have always pushed me to be the best that I can be and without your support I would have never succeeded in high school, college, graduate school, and in life. I am so proud and thankful to be your daughter. All I ever needed was your love, but both of you have given me so much more! Ryan, I know that the age difference made it hard for us to be close growing up and the distance didn’t help either, but I want you to know that I will always be here for you and that I hope we can grow closer in the years to come. You have brought a lot of laughs to our family and I hope that never changes. Your journey is just beginning and I can’t wait to see who you become! I love you all, and thank you for loving me!

“Other things may change us but we start and end with family.” – Anthony Brandt

F.R.O.G.
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I have dedicated this dissertation to my parents, however, I can never thank them enough, so thank you again for all that you are and all that you do! I would also like to thank all of my other family members (aunts, uncles, cousins, etc.) for their love and support.

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Abstract

Arginine methylation is catalyzed by the protein arginine methyltransferase (PRMT) family of enzymes, which transfer a methyl group from S-adenosylmethionine (SAM) to the guanidinium group of an arginine residue. This reaction first produces monomethylated arginine (MMA) that can then be further methylated to produce either asymmetrically dimethylated arginine (ADMA) or symmetrically dimethylated arginine (SDMA). There are nine PRMT family members described to date, with PRMT1 as the predominant member, suggested to be responsible for \(~85\%\) of asymmetric dimethylation. In addition, PRMT1-dependent methylation likely plays a significant role in a plethora of diseases (e.g., cancer, heart disease, and ALS). These observations render it imperative that the isozyme be more thoroughly characterized and suggests that potent and selective inhibitors may be useful as therapeutics.

Herein we describe our efforts to decode PRMT1-dependent methylation by investigating the catalytic mechanism, the effects of post-translational modifications and protein-protein interactions on activity, the development of potent and selective inhibitors and inactivators, as well as examining crosstalk between arginine methylation and phosphorylation. Using site-directed mutagenesis and unnatural amino acid incorporation, we have identified key active site residues that are critical for catalysis and/or substrate binding, and have determined the effects of phosphorylation, if any, on enzyme activity. In vitro assays with known interacting proteins has increased our
knowledge of the regulation of PRMT1 activity by protein-protein interactions. The use of MS/MS analysis aided in the identification of the site of modification for a potent inactivator of the isozyme, C21, and has led to the design of new inhibitors and inactivators that will likely be more potent and selective for not only PRMT1, but PRMT5 as well. Finally, using a peptide based model, we began to investigate crosstalk between arginine methylation and serine/threonine phosphorylation within kinase consensus sequences and hypothesize that it is an important means of regulation in regards to cell signaling. Overall, the results presented in the following chapters have enhanced our understanding of PRMT1-dependent methylation and have opened doors for future studies involving the regulation of the enzyme and inhibitor design.
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CHAPTER 1

Introduction

1.1 Post-Translational Modifications

Post-translational modifications (PTMs) of proteins are well-known for the variety of roles they play in controlling cellular functions. With over 400 different experimentally determined types (Khoury et al. 2011), PTMs add to the diversity of the already complex nature of the proteome by influencing protein-protein interactions, the cellular location of proteins, and protein stability through alterations to the size, charge, and hydrogen bonding capabilities of the parent residues (Figure 1.1). These processes are the basis for a plethora of cellular functions (e.g., transcription and signal transduction) that are vital to the maintenance, growth, and survival of healthy cells. Due to their fundamental roles in the cell, the dysregulation of various PTMs has been associated with a wide range of diseases (e.g., cancer and autoimmune diseases), thus making the responsible enzymes attractive drug targets.

1.2 Modification of Histones

The most notable and well-studied group of modified proteins are the histones. In the nucleus, two of each of the four core histones (i.e., H2A, H2B, H3, and H4) come together as an octamer around which DNA is wrapped and held in place by histone H1 to form a complex known as the nucleosome. Nucleosomes are further packaged into chromatin, and it is the state of the chromatin (i.e., decondensed vs. condensed) that
Figure 1.1 Selected post-translational modifications of arginine, lysine, serine, threonine, and tyrosine. (A) Arginine residues can be mono- and dimethylated by the PRMTs to form ω-MMA, ADMA, or SDMA. They can also be converted to citrulline by the PADs. (B) Lysine residues can be mono-, di-, and trimethylated by KMTs, acetylated by KATs, or ubiquitinated by ubiquitin ligases. (C) Serine, threonine, and tyrosine residues can be phosphorylated by kinases.
dictates whether genes are transcribed. PTMs on the unstructured N-terminal tails of histones are responsible for switching the transcription of genes on and off using what has been termed the histone code (Figure 1.2). For example, specific modifications can

![Figure 1.2 Post-translational modifications of histones.](image)

cause the destabilization of chromatin via the disruption of key interactions between DNA and histones (i.e., electrostatic interactions), such as in the case of acetylation of lysine 16 on histone H4 and the inhibition of heterochromatin formation (Shogren-Knaak et al. 2006 & Bannister et al. 2011). They can also aid in the recruitment of the transcriptional machinery through protein domains that are capable of binding such PTMs (e.g., bromo-, chromo-, tudor-, and pleckstrin-homology (PH) domains (reviewed in Kouzarides et al. 2007). In terms of blocking transcription, a modification can also recruit a particular protein that stabilizes chromatin and silences genes, as in the case of
tri-methylated lysine 9 of histone H3 and its recruitment of heterochromatin protein 1 (HP1) (Canzio et al. 2011).

1.3 Crosstalk Between Post-Translational Modifications

Over the last decade there have been several examples of crosstalk between two or more different post-translational modifications (PTMs), with many of these being observed within the context of histones. Generally, this crosstalk is thought to modulate and fine-tune cell signaling cascades such that a desired outcome is achieved e.g., transcription of a particular gene or, alternatively, activation of one gene under the control of a transcription factor and repression of another. Although crosstalk between two or more PTMs has predominantly been studied within the context of chromatin biology (Suganuma et al. 2008 and Lee et al. 2010), as one would expect, this type of regulatory mechanism extends to non-histone proteins as well. Several models have been proposed for histone crosstalk (Fischle 2003 et al., Schreiber et al. 2002, Fischle et al. 2008), and they are readily applied to non-histone proteins as well (Figure 1.3). For example, cis crosstalk refers to communication between modifications on the same protein (Figure 1.3A). Within cis crosstalk lies the possibility for adjacent crosstalk (i.e., between residues that are close to one another in both the primary and tertiary structures) or distal crosstalk (i.e., between residues that are far apart in both the primary and tertiary structures) (Figure 1.3A). Trans crosstalk is also possible and occurs between modifications on two different proteins (Figure 1.3B). Functionally, direct crosstalk refers to one PTM directly affecting the modification of a second residue (e.g., modification of one residue prevents the modification of another residue) (Figure 1.3C).

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1 Adapted with permission from Rust, H.L.; Thompson, P.R., Kinase consensus sequences: a breeding ground for crosstalk. ACS chemical biology 2011, 6 (9), 881-892. Copyright 2013 American Chemical Society.
Figure 1.3 Crosstalk scenarios. (A) The cis-effect refers to crosstalk between two or more modifications located on the same protein. Within the same protein there can be adjacent (i.e., between residues that are close in both primary and tertiary structures) or distal (i.e., between residues that are separated in primary and tertiary structures) crosstalk. (B) The trans-effect refers to crosstalk between two modifications located on two different proteins. Functionally, crosstalk can be (C) direct (i.e., one modification inhibits or enhances the subsequent modification of the same or a different residue) or (D) indirect (i.e., a specific modification inhibits or enhances protein-protein interactions leading to altered downstream effects). Note that while this figure depicts crosstalk involving histones, the same explanations can be applied to non-histone proteins. Adapted from Fischle et al. 2008.
Indirect crosstalk involves modulating a protein-protein interaction via the presence, or lack, of a PTM (e.g., a PTM enhances the binding of a transcription factor leading to the recruitment of other coactivators) (Figure 1.3D). An early example of direct cis crosstalk (Figure 1.3A) from the histone field involves the phosphorylation of H3S10 and the acetylation of H3K14. Here, stimulation of the Ras-MAPK (mitogen activated protein kinase) pathway (Chadee et al. 1999) results in the Rsk-2 (ribosomal S6 kinase) dependent phosphorylation of H3S10 (Sassone-Corsi et al. 1999), which enhances the acetylation of H3K14 by generating a better substrate for the histone acetyltransferase Gcn5 (general control non-repressed 5) (Cheung et al. 2000, Clayton et al. 2000, and Lo et al. 2000). Although this is only one example of crosstalk from the histone field, a plethora of others have been published (reviewed in Suganuma et al. 2008, Lee et al. 2010, and Baek et al. 2011), including several papers that describe crosstalk in non-histone proteins, with a particular set of crosstalk examples involving serine/threonine phosphorylation and the modification of neighboring arginine residues; and these examples will be discussed later in Chapter 6.

1.4 Arginine Modifications

Arginine residues within proteins can undergo several different types of PTMs, some of which are more prevalent and well-studied than others. These modifications include enzyme mediated modifications, i.e., citrullination, methylation, phosphorylation, and ADP-ribosylation, as well as non-enzymatic modifications, i.e., advanced glycation end-products (AGE) and carbonylation (reviewed in Slade et al. 2013). The most relevant modifications to this thesis are discussed in greater detail below.
1.4.1 Citrullination

Citrullination, also known as deimination, is catalyzed by the protein arginine deiminase (PAD) family of enzymes, which is comprised of PADs1-4 and PAD6. These isozymes catalyze the conversion of the guanidium moiety of arginine to an ureido moiety via a calcium dependent hydrolytic mechanism (Kearney et al. 2005). This PTM alters electrostatic interactions by changing a positively charged residue to a neutral residue (Figure 1.4). In regards to histones, the in vivo sites of citrullination have been determined to be H2 Arg 3 (Hagiwara et al. 2005), H3 Arg 2, Arg 8, Arg 17 (Cuthbert et al. 2004), and Arg 26 (Cuthbert et al. 2004 & Zhang et al. 2012), and H4 Arg 3 (Wang et al. 2004). Citrullination of these sites correlate with either transcriptional repression (e.g., citrullination of H3 Arg 17 at the pS2 promoter) (Cuthbert et al. 2004, Wang et al. 2004) or activation (e.g., citrullination of H3 Arg 26 at ERα target genes) (Zhang et al. 2012), depending upon the specific histone and residue.

![Figure 1.4 PAD catalyzed reaction](image)

Figure 1.4 PAD catalyzed reaction. PADs catalyze the conversion of the guanidinium moiety of an arginine residue to an ureido moiety via a calcium dependent hydrolytic mechanism.

In addition to its role in transcription, histone citrullination, specifically histone H3 by PAD4, is essential for neutrophil extracellular trap (NET) formation, a component
NETs are comprised of decondensed chromatin, with the DNA and histones acting as traps for pathogens (Figure 1.5) (Neeli 2008 et al., Wang et al. 2009, Li et al. 2010). Non-histone proteins, such as myelin basic protein (MPB) (Wood et al. 2008) and antithrombin (Chang et al. 2005), are also citrullinated by members of the PAD family, thus demonstrating the versatility of these isozymes. Citrullination has become an increasingly important PTM because of its apparent roles in diseases such as Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), ulcerative colitis, Alzheimers Disease (AD), and cancer (reviewed in Jones et al. 2009). In fact, Cl-amidine, a pan-PAD inhibitor, was found to decrease disease severity in animal models of spinal cord injury (Lange et al. 2011), collagen-induced arthritis (CIA) (Willis et al. 2011), ulcerative colitis (Chumanevich et al. 2011), and cancer (McElwee et al. 2012).

Figure 1.5 Role of PAD4 in NET formation.
1.4.2 Methylation

Methylation of arginine residues is catalyzed by the protein arginine methyltransferase (PRMT) family of enzymes. These isozymes transfer a methyl group from S-adenosylmethionine (SAM) to the guanidinium moiety of arginine residues in proteins, but not free arginine. This reaction first produces an \( \omega \)-monomethylarginine residue (\( \omega \)-MMA), which in most cases is further methylated to produce either an asymmetrically dimethylated arginine residue (ADMA) or a symmetrically dimethylated arginine (SDMA) residue (Figure 1.6). The addition of one or two methyl groups does not alter the charge of the residue; however, it decreases the number of potential hydrogen bond donors, thus leaving the possibility of decreased intra- or intermolecular interactions. Unlike lysine methylation, this modification appears to be irreversible, as an

![Diagram of PRMT catalyzed reactions](image-url)
arginine demethylase has yet to be discovered. Some have suggested the possibility that PAD4 may catalyze a demethylimination reaction that would convert methylated arginine to citrulline. This conversion would not truly reverse the modification but may have a similar function. There is conflicting evidence \textit{in vitro} and \textit{in vivo} however, as to whether this reaction actually occurs in the cell, with more evidence supporting the notion that it does not take place (reviewed in Thompson et al. 2006).

\subsection*{1.5 Protein Arginine Methyltransferase Family}

In humans, there are nine PRMT family members including: PRMT1, -2, -3, -4, -6, and -8 (Yang et al. 2013), which are type I PRMTs that produce ADMA; PRMT5, which is a definitive type II PRMT and produces SDMA (Yang et al. 2013); and PRMT7, which is a type III PRMT and generates only \(\omega\)-MMA (Miranda et al. 2004 & Zurita-Lopez et al. 2012) (Figure 1.6). Note that enzymatic activity has yet to be demonstrated for PRMT9. All PRMTs possess a highly conserved \textasciitilde310 amino acid catalytic core that is responsible for methyltransferase activity. This core consists of a SAM binding domain that contains a Rossmann type fold typical of Class I methyltransferases, a unique \(\beta\)-barrel domain, and a dimerization arm. All family members possess an N-terminal extension and several also contain C-terminal extensions (Figure 1.7) (reviewed in Yang et al. 2013).

\subsubsection*{1.5.1 PRMT1}

PRMT1 is the most prevalent PRMT isozyme and it is thought to be responsible for \textasciitilde85\% of the asymmetrically dimethylated arginine residues \textit{in vivo} (Tang et al. 2000 & Pawlak et al. 2000). The PRMT1 gene, located at 19q13.3 in humans (Scorilas et al. 2000), is highly conserved with \textasciitilde50\% sequence identity between humans and \textit{S.}
Figure 1.7 Structure of PRMT family members. PRMT family members have four common motifs in their SAM binding domain and one motif in their unique PRMT domain. Each isozyme has a distinct N-terminus with some containing common protein domains such as a SH3 or a Zn finger domain. Adapted from Yang et al. 2013.

cerevisiae and over 90% between mammals, zebrafish, and Xenopus (Zhang et al. 2003).

This isozyme was originally discovered as an interacting partner of the immediate-early gene TIS21 (Lin et al. 1996), leukemia-associated BTG1 (Lin et al. 1996), and interferon-α receptor (IFNAR1) (Abramovich et al. 1997), as well as via sequence homology to a yeast homolog (Scott et al. 1998). PRMT1 is the smallest member of the PRMT family and has three major human splice variants (i.e., PRMT1v1-v3) (Scott et al. 1998 & Goulet et al. 2007), that translate into proteins ranging from 353-371 amino acids in length (Scott et al. 1998 & Pawlak et al. 2000), and four minor variants (i.e., PRMT1v4-v6 (Zhang et al. 2003 & Goulet et al. 2007) and v7 (Goulet et al. 2007)). Although we
are only beginning to understand the differences between these splice variants, the variation of which lies within the N-terminus (Figure 1.8), these variants show different tissue expression patterns and have effects on subcellular localization. For example, PRMT1 is expressed in all tissues studied thus far (Scott et al. 1998, Tang et al. 1998, Lin et al. 1996, Pawlak et al. 2000), with PRMT1v1 and –v2 found in several tissues, -v4 only in the heart, -v5 predominately in the pancreas, no detection of –v6, and -v7 mostly in the heart and skeletal muscles (Goulet et al. 2007). In regards to subcellular localization, PRMT1 as a whole is located in both the nucleus and the cytoplasm (Tang et al. 1998, Cote et al. 2003, Frankel et al. 2002, Goulet et al. 2007). More specifically, PRMT1v3, -v4, -v5, and –v6 are diffuse throughout the cell (Goulet et al. 2007), whereas PRMT1v1 and –v7 are more nuclear (Goulet et al. 2007) and PRMT1v2 is primarily cytoplasmic (Herrmann et al. 2005 and Goulet et al. 2007). In fact, PRMT1v2 is the only variant that contains a nuclear export signal (Goulet et al. 2007).
2007) and was found to translocate from the nucleus, contingent on substrate methylation status (Herrmann et al. 2005) and catalytic activity of the enzyme (Herrmann et al. 2009). Interestingly, these different N-terminal tails are also important for the substrate specificities of PRMT1, as demonstrated by the different methylation profiles observed for each of the variants. For example, known PRMT substrates such as SmB and Sam68 were methylated to a greater extent by PRMT1v1 and –v2, but hnRNP A1 was a better substrate for PRMT1v5 and -6 (Goulet et al. 2007). In addition, incubation of purified PRMT1 variants with extracts prepared from mouse embryonic stem cell yielded distinct visual differences in the proteins methylated by PRMT1v1 and –v2 (Goulet et al. 2007). Similar results were also observed with purified mouse PRMT1v1 and –v2 and mouse embryonic stem cell extracts (Pawlak et al. 2002). Surprisingly, the addition of N-terminal His6 tags to the two main variants abolished the differences in substrate specificity (Pawlak et al. 2002), thus demonstrating the uniqueness of the N-termini of PRMT1. It is hypothesized that the unstructured N-terminal tail of the enzyme folds back and interacts with its substrates, which would account for the differences in observed substrate specificity (Goulet et al. 2007).

Although vital for early postimplantation development, PRMT1 is not essential for cell viability (Pawlak et al. 2000). This isozyme is involved in transcriptional regulation through both its methylation of Histone H4 at arginine 3 (Strahl et al. 2001 & Wang et al. 2001) and other proteins involved in transcription (e.g., transcription factors (Yamagata et al. 2008 & Jobert et al. 2009), coactivators (Teyssier et al. 2005), elongation factors (Kwak et al. 2003)), and RNA binding proteins (Cote et al. 2003, Ostareck-Lederer et al. 2006, Chiou et al. 2007). It primarily acts as a coactivator for
nuclear receptors (Koh et al. 2001) and transcription factors (i.e., YY1 (Rezai-Zadeh et al. 2003), p53 (An et al. 2004), STAT5 (Kleinschmidt et al. 2008), and AE9a (Shia et al. 2012)), however, there are instances in which PRMT1 represses transcription (Kleinschmidt et al. 2008). Interestingly, it was determined that methylation of arginine 3 of Histone H4 enhances acetylation of lysine residues on both Histone H3 (Huang et al. 2005) and Histone H4 (Wang et al. 2001 & Huang et al. 2005), but prior acetylation prevents methylation (Huang et al. 2005). PRMT1 also plays a variety of other roles in the cell. More specifically, it is involved in insulin signaling (Iwasaki et al. 2007), estrogen signaling (Le Romancer et al. 2008), and interferon signaling (Abramovich et al. 1997), as well as, DNA damage response pathways through methylation of MRE11 (Boisvert et al. 2005, Dery et al. 2008, Yu et al. 2009) and 53BP1 (Boisvert et al. 2005).

1.5.2 PRMT2

PRMT2, the gene of which is located at 21q22.3, was discovered by Katsanis et al. and contains 57% nucleotide sequence homology to PRMT1 (Katsanis et al. 1997). The translated protein is 433 amino acids in length with an N-terminal extension that distinguishes it from PRMT1 (Krause et al. 2007). This extension contains a SRC Homology 3 (SH3) domain, which is known to facilitate protein-protein interactions (Pawson et al. 1992, Mayer et al. 1993). Interestingly, the SH3 domain is required for PRMT2’s interaction with E1B-AP5 (Kzhyshkowska et al. 2001) but not estrogen receptor alpha (ERα) (Qi et al. 2002). Originally, methyltransferase activity for this enzyme could not be detected directly (Kzhyshkowska et al. 2001, Qi et al. 2002, Ganesh et al. 2006, Meyer et al. 2007). It was recently discovered, however, that PRMT2 is a Type I PRMT (Lakowski et al. 2009) and is capable of methylating recombinant histone
H4 *in vitro* (Lakowski et al. 2009) and histone H3R8 in *Xenopus* (Blythe et al. 2010). PRMT2 appears to mainly play the role of a transcriptional co-activator for a number of nuclear hormone receptors (e.g., androgen receptor, estrogen receptor) (Meyer et al. 2007, Qi et al. 2002), which is unexpected because it is a cytoplasmic protein. However, evidence has shown that it can be transported into the nucleus with the androgen receptor upon hormone stimulation (Meyer et al. 2007).

### 1.5.3 PRMT3

PRMT3 is a Type I PRMT that was identified in a yeast two-hybrid screen for PRMT1 interacting proteins (Tang et al. 1998). The gene is located at 11p15.1 and encodes a 531 amino acid protein that contains an N-terminal zinc finger domain (Krause et al. 2007). This zinc finger domain was found to dictate substrate specificity and is vital for PRMT3’s interaction with RNA-associated proteins (Frankel et al. 2000). PRMT3 is located in the cytoplasm and, although the crystal structure suggests that it can form homodimers (Zhang et al. 2003), it was found as a monomer using gel filtration of rat cell extracts (Tang et al. 1998). The major substrate of PRMT3 is the 40 S ribosomal protein S2 (rpS2), thus suggesting that this enzyme may play roles in the regulation of protein synthesis and or ribosome assembly (Swiercz et al. 2005).

### 1.5.4 PRMT4/CARM1

PRMT4, more commonly known as coactivator-associated methyltransferase 1 (CARM1), was discovered during a yeast two-hybrid screen for proteins that interact with the AD2 domain of p160 coactivators (Chen et al. 1999). The gene is located at 19p13.2 and encodes a 608 amino acid protein in humans (Krause et al. 2007). Although most PRMTs only contain a unique N-terminal extension, CARM1 contains both an N-
and C-terminal extension. Neither of these extensions are required for enzymatic activity, homo-oligomerization, or p160 binding in vitro (Teyssier et al. 2002), however, both of them are required for the isozyme’s transcriptional coactivator function. The N-terminus adopts a pleckstrin homology domain (PH) fold that is typically involved in the formation of multiprotein complexes and the regulation of protein-protein interactions. The role of that this fold plays in the function of this isozyme remains to be determined (Troffer-Charlier et al. 2007). Importantly, it was demonstrated that CARM1 is essential to life in that knockout mice die shortly after birth (Yadav et al. 2003). CARM1 is known to methylate Histone H3 at arginine 2, 17, and 26 (Schurter et al. 2001), several splicing factors (e.g., SmB and U1C) (Cheng et al. 2007), as well as transcriptional co-activators (e.g., SRC-3 (Feng et al. 2006), CBP (Xu et al. 2001 and Chevillard-Briet et al. 2002), and p300 (Chevillard-Briet et al. 2002). The identities of these CARM1 substrates exemplify the role of this isozyme as a coactivator involved in transcriptional regulation (Ma et al. 2001, Schurter et al. 2001, Chevillard-Briet et al. 2002, Feng et al. 2006).

1.5.5 PRMT5

Human PRMT5, originally known as Jak-binding protein 1 (JBP1), was identified during a search for Janus kinase 2 (Jak2) interacting proteins (Pollack et al. 1999) and was found to be a homologue of the previously reported Skb1 from Schizosaccharomyces pombe (Gilbreth et al. 1996) and HSL7 from Saccharomyces cerevisiae (Ma et al. 1996). It is the only known Type II PRMT and produces SDMA (Branscombe et al. 2001). Located at 14q11.2, the PRMT5 gene encodes a 637 amino acid protein (Krause et al. 2007) with a large N-terminal extension that contains a TIM barrel domain (Antonysamy et al. 2012). This TIM barrel domain is solely responsible for the interaction of this
isozyme with its binding partner methylosome protein 50 (MEP50) (Antonysamy et al. 2012). MEP50 is a WD40-repeat containing protein that is required for PRMT5 methyltransferase activity and aids in substrate recognition and interactions with other proteins, as the isozyme shows minimal activity unless it is a part of a larger multiprotein complex (Friesen et al. 2002). Located in the nucleus and the cytoplasm, PRMT5 plays roles in a plethora of cellular processes such as transcriptional regulation (Pal et al. 2004, Pal et al. 2007, Wang et al. 2008), differentiation (Dacwag et al. 2007, Dacwag et al. 2009, Mallappa et al. 2011), the synthesis of ribosomes (Ren et al. 2010), and cell proliferation (Pal et al. 2007 & Wang et al. 2008). Similar to PRMT1, PRMT5 is essential for both embryonic development and the derivation of embryonic stem cells (Tee et al. 2010).

1.5.6 PRMT6

Discovered during a human genome search for PRMTs (Frankel et al. 2002), the PRMT6 gene is located at 1p13.1 and encodes a 375 amino acid protein (Krause et al. 2007). A type I PRMT, this particular isozyme is located exclusively in the nucleus and was the first PRMT discovered to be capable of automethylation (Frankel et al. 2002). PRMT6 plays roles in transcriptional regulation, regulation of HIV replication, base excision repair, and cell cycle progression via methylation of histone H2A arginine 3 (Hyllus et al. 2007) and arginine 29 (Waldmann et al. 2011), histone H3 arginine 2 (Hyllus et al. 2007, Guccione et al. 2007, Iberg et al. 2008), histone H4 arginine 3 (Hyllus et al. 2007), HIV-1 Tat (Boulanger et al. 2005), HMGAla/b (Miranda et al. 2005 and Sgarra et al. 2006), DNA polymerase β (El-Andaloussi et al. 2006), and tumor suppressor p16 (Wang et al. 2012). In addition, our lab recently determined the kinetic mechanism
of PRMT6 to be a rapid equilibrium random mechanism with dead end EAP and EBQ complexes (Obianyo et al. 2012).

1.5.7 PRMT7

The PRMT7 gene, which was also discovered from computational alignments of potential PRMT genes (Miranda et al. 2004 and Lee et al. 2005), is located at 16q22.1 (Krause et al. 2007). The gene encodes a 692 amino acid protein (Krause et al. 2007) that uniquely contains an additional but less conserved SAM binding domain at the C-terminus (Miranda et al. 2004). Surprisingly, the presence of both SAM binding domains were found to be necessary for methyltransferase activity, although SAM could only crosslink to the N-terminal SAM binding domain (Miranda et al. 2004), suggesting that only the N-terminal half of the enzyme is active and the C-terminus acts as a regulatory domain. Although originally there were conflicting results regarding the final product of methylation, with one group suggesting a Type III enzyme and only the formation of ω-MMA (Miranda et al. 2004) and another group suggesting a Type II enzyme and the formation of both ω-MMA and SDMA (Lee et al. 2005), recent evidence has supported the notion that it is a Type III enzyme with the latter result possibly being an artifact of contamination by PRMT5 (Zurita-Lopez et al. 2012). Like PRMT1, PRMT7 is located in both the nucleus and cytoplasm (Lee et al. 2005). Although one of the lesser characterized PRMTs, it has been suggested that this isozyme plays a role in cellular differentiation (Buhr et al. 2008) and the DNA damage response (Gros et al. 2006 and Verbiest et al. 2008).

1.5.8 PRMT8

Human PRMT8, located on chromosome 12p13.3 and encoding a 394 amino acid
protein, was originally discovered due to its 80% sequence homology to PRMT1, with the major variations located at the N-termini (Zhang et al. 2003). Despite the high sequence homology between the two isozymes, PRMT8 expression is primarily restricted to brain tissue (Lee et al. 2005, Taneda et al. 2007, Kousaka et al. 2009) and it is targeted to the plasma membrane via myristoylation of its N-terminus at glycine-2 at its N-terminus (Lee et al. 2005). Interestingly, the N-terminus also plays a regulatory role in enzymatic activity in that the full length recombinant protein displays significantly decreased activity compared to a N-terminal truncated variation that more closely resembles PRMT1 (Sayegh et al. 2007). The isozyme is also capable of automethylation via the production of ADMA on arginine 73 and ω-MMA on arginine 58, however, it is still uncertain as to whether this is an intra- or inter- molecular reaction, as PRMT8 is capable of forming homodimers (Sayegh et al. 2007). Additionally, two proline rich sequences on the N-terminus have been found to bind SH3 domains of proteins, including the SH3 domain of PRMT2, however, the functional significance of these interactions is unknown as no change in PRMT8 activity was observed in in vitro assays (Sayegh et al. 2007). Several PRMT8 interacting proteins (e.g., TET-family of RNA-binding proteins, hnRNPs, and actin) were revealed via in vitro GST-pull down experiments with recombinant GST-PRMT8 and hypomethylated cell extracts. Specifically, the isozyme co-localizes with Ewing’s sarcoma (EWS), independent of the binding partner’s methylation status (Pahlich et al. 2008). The function of PRMT8 in the cell remains to be fully defined.

1.5.9 PRMT9

PRMT9, often denoted along with its chromosomal location (4q31), was
identified by sequence homology with other PRMT family members (Lee et al. 2005). No enzymatic activity has been reported and it has yet to be characterized. Sequence analysis has shown that it is most closely related to PRMT7 in that it contains a second SAM binding domain on the C-terminus. The N-terminus contains two tetratricopeptide repeats, which based on the previously reported functions of this motif (Blatch et al. 1999), could potentially play a role in protein-protein interactions (Bedford et al. 2007).

A controversy remains in regards to a second PRMT9, PRMT9 (2p16), which is also known as F-box only protein 11 (FBXO11). This enzyme was found to produce ω-MMA, ADMA, and SDMA (Cook et al. 2006) despite being structurally different from the PRMTs, which are a part of the Type I seven-β strand methyltransferase family (Katz et al. 2003 & Bedford et al. 2009). A separate study demonstrated that this protein had no methyltransferase activity (Fielenbach et al. 2007); the production of SDMA in the original report may possibly be due to sample contamination by PRMT5 (Nishioka et al. 2003). Therefore, the methyltransferase classification of this protein has yet to be confirmed.

1.6 Role of PRMT1 in Disease

With the multitude and variety of roles that the PRMT isozymes play in the cell, it is conceivable that their dysregulation would be involved in the pathogenesis of one or more human diseases. Our research thus far has focused on PRMT1 because it is the major Type I methyltransferase and would therefore logically play a greater role in the onset and progression of diseases compared to the other isozymes. The following subsections will give an overview of the involvement of PRMT1 in various diseases.
1.6.1 Cancer

A recent study of the expression levels of PRMT1 in tumors from various tissues revealed that the isozyme, and in some cases select splice variants, is/are significantly overexpressed in a variety of cancers (Yoshimatsu et al. 2011). The results of this study are in agreement with several more specific studies that have shown that PRMT1 is overexpressed in breast cancer (Goulet et al. 2007)(Baldwin et al. 2012), colon cancer (Mathioudaki et al. 2008), gliomas (Wang et al. 2012), acute lymphoblastic leukemia (ALL) (Zou et al. 2012). In addition, higher levels of serum ADMA is observed in cancer patients, an observation that is the first of its kind (Yoshimatsu et al. 2011).

1.6.1.A Breast Cancer

Estrogen and its receptor, estrogen receptor α (ERα), are well-known for the genomic roles that they play in breast cancer, demonstrated by the fact that 70% of breast cancers are estrogen dependent and ERα positive (Le Romancer et al. 2008). Surprisingly, evidence suggests that the role that PRMT1 plays in breast cancer is of nongenomic origin. In the cytoplasm, the DNA binding domain of ERα is methylated by PRMT1 at arginine 260, in response to estrogen. This methylation event leads to the formation of a multiprotein complex involving the receptor itself, Src kinase, phosphoinositide 3-kinase (PI3K), and focal adhesion kinase (FAK). This complex ultimately activates protein kinase B (Akt) and induces cell proliferation and survival (Figure 1.9) (Le Romancer et al. 2008). Interestingly, in the same study, 55% of invasive breast cancer tissues analyzed had high levels of methylated ERα and 45% had low levels (Le Romancer et al. 2008), thus demonstrating a plausible nongenomic role for PRMT1 in breast cancer.
A second nongenomic role for PRMT1 in breast cancer has also been revealed (Figure 1.10). The authors of this study had previously shown that the different splice variants of PRMT1 are overexpressed in breast cancer cells to varying degrees, with PRMT1v2 having a more significant increase than the predominant PRMT1v1 (Goulet et al. 2007), which led them to further investigate the contribution of this particular variant to the disease. RNA interference was used to abolish PRMT1v2 expression and yielded an increase in apoptosis and decreased cell invasion in an aggressive cell line. Intriguingly, overexpression of this variant caused increased invasiveness in a known non-aggressive cell line, that was not observed with the other PRMT variants. The role of PRMT1v2 in cell motility and invasion is dependent on the localization and activity of the enzyme as mutations to the NES and activity site abolish the effects. Further investigation into the role of this particular variant showed that it is likely involved in regulating β-catenin degradation, a protein involved in cell-cell adhesion, as an increase in phosphorylated β-catenin was observed in PRMT1v2 expressing cells (Baldwin et al. 2012) (Figure 1.10). This observation is in concordance with and in addition to another study that demonstrated a role for PRMT1 in controlling β-catenin expression in the
cytoplasm through regulation of Wnt signaling via methylation of Axin (Cha et al. 2011).

Figure 1.10 Proposed model of the role of PRMT1v2 in breast cancer. (A) In the absence of PRMT1v2, the Wnt signaling pathway targets Axin to the membrane, which allows β-catenin to enter the nucleus. (B) In the presence of PRMT1v2, Axin is methylated, which leads to the phosphorylation of β-catenin and subsequent degradation (Cha et al. 2011 & Baldwin et al. 2012).

1.6.1.B Leukemia

The mixed lineage leukemia (MLL) gene is commonly associated with ALL and AML in that chromosomal translocation of the gene forms fusion proteins that aberrantly regulate and activate the expression of genes such as class I homeobox (HOX) (Daser et al. 2005) and ultimately transform early myeloid progenitors and haematopoietic stem cells (Cozzio et al. 2003 & So et al. 2003). PRMT1 was discovered to be a critical component of the MLL transcriptional complex comprised of the SH3-domain containing MLL-EEN oncogenic fusion protein, SAM68, an RNA-binding protein and substrate of PRMT1, and CREB-binding protein (CBP), a histone acetyltransferase (Cheung et al. 2007). Results of several in vitro and in vivo experiments demonstrate that MLL-EEN
binds SAM68 via its SH3-domain which leads to the recruitment of both PRMT1 and CBP to downstream targets of the fusion protein, in this case *Hoxa9a* (Figure 1.11). Methylation of histone H4 arginine 3 by PRMT1 leads to increased acetylation of histone tails by CBP and activation of gene transcription (Cheung et al. 2007). This observation is in agreement with another study demonstrating cooperativity between PRMT1 and p300, a histone acetyltransferase related to CBP (An et al. 2004). Other studies have shown that CBP interacts with wild type MLL (Ernst et al. 2001 & Daser et al. 2005) but recruitment of CBP alone by another MLL fusion protein, MLL-AFX, did not induce cellular transformation (So et al. 2002), thus suggesting that other factors are necessary. On the other hand, PRMT1 does not interact with wild type MLL, but the creation of a MLL-PRMT1 fusion protein transformed primary myeloid progenitor cells, thus demonstrating that the PRMT1 alone plays a key role in the MLL dependent leukemia (Cheung et al. 2007).

In a separate but similar example, PRMT1 interacts with a splice form (AE9a) of AML1-ETO, a transcription factor fusion protein associated with AML. This particular
isoform was identified from patient samples and rapidly induces leukemia in a mouse model (Shia et al. 2012). Recruitment of PRMT1 by AE9a to the promoters of target genes yields activation of the genes by methylation of histone H4 and subsequent histone acetylation (Shia et al. 2012), as observed in in the previous example. To further demonstrate the importance of PRMT1 in this pathway, knockdown of the enzyme decreased proliferation of AE9a leukemic cells (Shia et al. 2012).

### 1.6.2 Heart Disease

Heart disease is the leading cause of death in the United States for both men and women (Hoyert et al. 2012) and PRMT1 may be a contributing factor. In addition to the observation that the enzyme is overexpressed in tissues of patients with this disease (Chen et al. 2006), free MMA and ADMA, which are products of the degradation of PRMT1 substrates, are competitive inhibitors of the nitric oxide synthases (NOSs), vital enzymes responsible for the production of nitric oxide (NO) (Valance et al. 1992). Free MMA and ADMA are normally converted to citrulline by dimethylarginine dimethylaminohydrolase (DDAH) and can then be further broken down and used for protein synthesis or excreted (Vallance et al. 2004) (Figure 1.12). An increase in PRMT1 expression coupled with dysfunction of DDAH can cause an increase in free MMA and ADMA (Vallance et al. 2004) and research efforts have focused on ADMA because its concentration is 10 times greater than MMA in human plasma (Vallance et al. 1992 & Tran et al. 2003). Elevation of free ADMA levels causes vasoconstriction (Achan et al. 2003 & Vallance et al. 2004), due to a decrease in NO production, and its levels are correlated with the severity of hypercholesterolemia and atherosclerosis, as well as the

1.6.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis is a progressive neurodegenerative disease that affects the entire motor system and half of patients die within 3 years of onset (Mitchel et al. 2007). In the familial form of ALS, which accounts for 10% of cases (Shaw et al. 1997), and more specifically termed ALS6, a gene fusion, i.e., fused in sarcoma/translocated in liposarcoma (FUS/TLS), was found to be mutated (Kwiatkowski et al. 2009 & Vance et al. 2009). FUS is normally involved in transcription, RNA processing, and translation as a ribonuclear protein (Law 2006 et al. & Wang et al. 2008) and plays a crucial role in neurons regarding the formation of dendritic spines (Fujii et al. 2005). Although WT FUS is found mostly in the nucleus, FUS mutants appear to be localized in the cytoplasm (Kwiatkowski et al. 2009, Vance et al. 2009, Bosco et al. 2010, Dormann et al. 2010, Gal et al. 2011, Ito et al. 2011, Kino et al. 2011) where they have been observed to form inclusions and associate with stress granules (Dormann et al. 2010, Bosco et al. 2010, Gal et al. 2011, Ito et al. 2011, Kino et al. 2011). Two recent studies have linked PRMT1 to ALS via FUS (Figure 1.13). Both WT (Yamaguchi et al. 2010, Bosco et al. 2010, Gal et al. 2011, Ito et al. 2011, Kino et al. 2011). Two recent studies have linked PRMT1 to ALS via FUS (Figure 1.13). Both WT (Yamaguchi et al. 2010, Bosco et al. 2010, Gal et al. 2011, Ito et al. 2011, Kino et al. 2011). Two recent studies have linked PRMT1 to ALS via FUS (Figure 1.13). Both WT (Yamaguchi et al. 2010, Bosco et al. 2010, Gal et al. 2011, Ito et al. 2011, Kino et al. 2011). Two recent studies have linked PRMT1 to ALS via FUS (Figure 1.13).
Figure 1.13 Proposed model of the role of PRMT1 in ALS. (A) In the absence and presence of PRMT1, WT FUS localizes in the nucleus. (B) In the absence of PRMT1, mutant FUS is capable of localizing in the nucleus, however, methylation of the mutant by PRMT1 leads to aggregation and formation of inclusions in the cytoplasm (Tradewell et al. 2012).

2012, Tradewell et al. 2012) and mutant FUS (Tradewell et al. 2012) are methylated by PRMT1, however, methylation appears to predominantly affect the function of mutant FUS, as demonstrated by a PRMT1 knockout model in mouse embryonic stem cells (MES) and HEK293 cells, as well as methyltransferase inhibition in motor neurons. The results revealed that PRMT1-dependent methylation appears to likely be a defining factor in the localization of mutant FUS to the cytoplasm (Tradewell et al. 2012). Interestingly, the ALS linked FUS mutations are present on the C-terminus of the protein where the nuclear localization signal (NLS) is also located and it is hypothesized that the mutations may affect nuclear-cytoplasmic shuttling. It remains unclear as to the exact role of the PRMT1-dependent methylation, however, it may enhance protein-protein interactions.
that ultimately lead to aggregation of mutant FUS in the cytoplasm and the formation of inclusions (Tradewell et al. 2012).

1.7 Conclusion

The involvement of PRMT1 in the aforementioned diseases demonstrates the likelihood that this enzyme is a worthwhile drug target. The close structural relationship between the PRMT family members presents a challenge in designing a selective inhibitor towards a particular isozyme. In addition, the presence of PRMT1 splice-variants with different substrate specificities and functions creates an additional level of difficulty. There is also a lack of knowledge at this time in regards to how enzyme activity is regulated (e.g., PTMs or protein-protein interactions). Herein, we will discuss mechanistic studies of PRMT1, potential methods of enzyme regulation, and the development of selective inhibitors. We will also present evidence for possible crosstalk between arginine methylation and phosphorylation.
CHAPTER 2
Mechanistic Studies on PRMT1

2.1 Introduction

PRMT1 shows the widest tissue distribution and highest expression, and is thought to be responsible for ~85% of the asymmetrically dimethylated arginine residues in vivo (Tang et al. 2000 & Pawlak et al. 2000). It is located in both the nucleus and the cytoplasm (Herrmann et al. 2005) and is active as a head-to-tail dimer, which is formed by the interaction of the dimerization arm of one monomer with the SAM binding domain of another monomer (Zhang et al. 2003). Our research thus far has focused on developing inhibitors that target this isozyme (Osborne et al. 2007, Osborne et al.2008, Obianyo et al. 2010, Bicker et al. 2010) due to its involvement in several diseases (e.g., cancer, heart disease, ALS). Previously, we demonstrated that PRMT1 preferentially methylates a 21 residue peptide based on the N-terminus of histone H4 with comparable kinetics to the parent protein (Osborne et al. 2007). Additionally, these studies demonstrated that positively charged residues present in the C-terminus of this peptide, which is denoted AcH4-21, are critical for the high rates of catalysis observed with this substrate. We further demonstrated that PRMT1 catalyzes the methylation of the AcH4-21 substrate in a partially processive manner, i.e., PRMT1 can rebind SAM and

2 Adapted with permission from Rust, H.L.; Zurita-Lopez, C.I.; Clarke, S.; Thompson, P.R., Mechanistic studies on transcriptional coactivator protein arginine methyltransferase 1. Biochemistry 2011, 50(16), 3332-45. Copyright 2013 American Chemical Society.
subsequently produce ADMA before the first methylation product, \( \omega \)-MMA, is released (Osborne et al. 2007). Because ADMA formation is not obligatory, we have suggested that PRMT1 displays partial processivity. The partially processive nature of this reaction is entirely consistent with the fact that PRMT1 uses a Rapid Equilibrium Random kinetic mechanism with dead-end E•SAM•\( \omega \)-MMA and E•AcH4-21•SAH complexes, where the E•SAM•\( \omega \)-MMA complex can undergo a second methyl transfer reaction to produce ADMA (Obianyo et al. 2008).

To follow up on these studies and provide a mechanistic basis for the methylation of an arginine residue, which is arguably a weak nucleophile, we examined the structure of PRMT1 bound to SAH (Zhang et al. 2003). Based on this structure, there are a number of highly conserved active site residues that likely play key roles in SAM recognition, substrate binding, and catalysis (Figure 2.1). For example, in PRMT1 it has been suggested that R54 and E100 are involved in SAM binding by hydrogen bonding and forming electrostatic interactions with the carboxylate group and ribose moiety of SAM, respectively (Zhang et al. 2000 & Zhang et al. 2003). The R54 residue also likely hydrogen bonds with the side chain of E144 to orient the \( \gamma \)-carboxylate of this residue for optimal electrostatic and hydrogen bond interactions with the N\( \eta_2 \) of a substrate arginine residue. This interaction likely helps position N\( \eta_2 \) for attack on the methyl group of SAM. The \( \gamma \)-carboxylate of E153 also likely contributes to the alignment of the substrate guanidinium via electrostatic and two hydrogen bond interactions with N\( \eta_1 \) and N\( \delta \) (Zhang et al. 2000 & Zhang et al. 2003), although, it should be noted that, in structures of PRMT1, the position of this residue does not appear to be catalytically competent as it is ‘flipped’ out of the active site (Zhang et al. 2003). Examination of the crystal structure of
CARM1 also identified Y154, a conserved tyrosine residue that corresponds to Y39 in PRMT1, as potentially playing a role in PRMT catalysis. Although Y39 is not visible in the crystal structure of PRMT1, the side chain phenol of this residue forms the top of the SAM binding pocket and is likely important for cofactor binding. Additionally, based on the CARM1 structure, the phenol appears to interact with E153 (PRMT1 numbering) and help orient this residue, and, as a consequence, the substrate guanidinium to promote catalysis (Yue et al. 2007 & Troffer-Charlier et al. 2007).

Figure 2.1 Active site of PRMT1. (A) Structure of PRMT1 (white) highlighting key residues in the active site believed to play roles in substrate binding and/or catalysis. Note that the PRMT1 structure is overlaid with PRMT3 (teal) because electron density of Y39 is not present in the crystal structure of PRMT1 and the positioning of E153 in PRMT1 is different from PRMT3, which is likely due to the crystallization conditions. This figure was prepared with UCSF Chimera using the coordinates from PRMT1 (PDBID 1ORI) and PRMT3 (PDBID 1F3L).
Also present in the active site is M155. Although this residue is not thought to play a direct role in rate acceleration, it has been suggested (Branscombe et al. 2001) that M155 is responsible for the formation of ADMA as the end product of dimethylation, as opposed to SDMA, due to steric hindrance that would prevent the transfer of a methyl group to Nη₁ after methylation of Nη₂ (Zhang et al. 2000 & Branscombe et al. 2001). This hypothesis is supported by the fact that PRMT5, a Type II PRMT, has a serine residue in this position that presumably creates a more open pocket that enables symmetric dimethylation (Branscombe et al. 2001).

Given that the guanidinium group is a relatively weak nucleophile, it has been suggested that its interaction with E153 causes a redistribution of electrons that activates Nη₂ for an S_N2-type nucleophilic attack on the methyl group of SAM (Figure 2.2) (Zhang et al. 2000). This attack potentially results in the formation of a dication intermediate that undergoes the loss of a proton to possibly E144 or via a proton wire to H293. However because the formation of a dication intermediate is somewhat unfavorable, it

![Figure 2.2 Proposed catalytic mechanism of PRMT1](image)

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has been suggested that PRMT1 uses a stepwise or concerted mechanism in which the proton is removed prior to or simultaneously with methyl transfer (Zhang et al. 2000).

Herein we describe our efforts to characterize the catalytic mechanism of PRMT1 using site directed mutagenesis on a number of highly conserved active site residues (i.e., Y39, R54, E100, E144, E153, M155, and H293), which are believed to play key roles in SAM recognition, substrate binding, and catalysis, as well as pH rate profiles, processivity studies, and the determination of Solvent Isotope Effects (SIEs).

2.2 Materials and Methods

2.2.1 Chemicals

Sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (TRIS), tetramethylethylenediamine, acrylamide, and ammonium persulfate were purchased from Bio-Rad (Hercules, CA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tricine, dithiothreitol (DTT) were purchased from RPI (Mt. Prospect, IL). Acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride and dimethylformamide (DMF) were purchased from Alfa Aesar (Ward Hill, MA). Piperidine was purchased from Sigma-Aldrich (St. Louis, MO). Fmoc protected amino acids, (ethylenedinitrilo)tetraacetic acid (EDTA), and trifluoroacetic acid (TFA) were purchased from EMD (Gibbstown, NJ). 14C-labeled SAM was purchased from Perkin-Elmer and 14C-labeled BSA from Sigma-Aldrich. Mutagenic primers were purchased from IDT Inc. (Coralville, IA).

2.2.2 Purification of PRMT1

The purification of PRMT1 has been described (Osborne et al. 2007). In brief, a pET28b construct (Zhang et al. 2003) containing the PRMT1 gene and an N-terminal
hexa-histidine tag was transformed into *E. coli* BL21(DE3) cells. One colony was used to inoculate 5 mL of LB media containing 50 μg/mL kanamycin and incubated overnight with shaking at 37 °C. Two liters of LB media were inoculated with 20 mL of overnight culture containing 50 μg/mL kanamycin and grown at 37 °C and 250 rpm until OD$_{600}$ = 0.4-0.6. Protein expression was then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside and the cells were incubated with shaking at 22 °C overnight. The next day the cells were harvested by centrifugation at 5000 rpm (4400 g) for 10 min. The pellet was resuspended in 30 mL of Lysis Buffer (20 mM HEPES pH 8, 100 mM NaCl, 5 mM imidazole pH 7.5, 0.5 mM PMSF, 5 mM 2-mercaptoethanol) and lysed using a French pressure cell at 20,000 psi. The lysate was centrifuged at 13,000 rpm (20,250 g) for 30 min and the supernatant was applied to a Ni$^{2+}$ Chelating Sepharose Fast Flow column. The protein was eluted using a 5 mM to 500 mM imidazole step gradient in 20 mM HEPES pH 8 and 100 mM NaCl. Fractions were screened on a 12% SDS-PAGE gel and fractions containing protein were dialyzed overnight in 20 mM HEPES pH 8.0 and 50 mM NaCl. The next day the protein was additionally purified by FPLC using a Mono Q anion exchange column (GE Healthcare). Fractions were screened on a 12% SDS-PAGE gel and fractions containing protein were dialyzed overnight in 100 mM HEPES, 200 mM NaCl, 1 mM DTT, 2 mM EDTA, and 10% glycerol. The next day the protein was concentrated using a 10 kDa Amicon Centriplus centrifugal filter and the concentration was determined using a Bradford assay. The enzyme was flash frozen in liquid nitrogen and stored at -80 °C.

**2.2.3 Site-Directed Mutagenesis**

PRMT1 mutants were generated using the QuikChange Site-Directed
Mutagenesis Kit™ (Stratagene). The full open reading frame was sequenced for each mutant to ensure that only the desired mutation had been incorporated. DNA that contained desired the mutation was then transformed into *E. coli* BL21(DE3) cells and purified using our established protocol for wild type (WT) PRMT1 (described above).

### 2.2.4 Synthesis of Peptides

AcH4-21 and RGG3 peptides were synthesized as previously described on a Rainin PS3 automatic peptide synthesizer using Fmoc chemistry on a Wang resin (Osborne et al. 2007). The sequences of these peptides can be found in Table 2.1. The peptides were cleaved from the resin with 95% TFA, 2.5% *triisopropylsilane*, and 2.5% water, and then precipitated with diethyl ether. Peptides were purified by reverse phase HPLC with a mobile phase of water/0.05% TFA and eluted with acetonitrile/0.05% TFA. The masses were determined using a Bruker Ultraflex II MALDI-TOF mass spectrometer.

### 2.2.5 Gel-Based Activity Assay

A previously described gel-based assay was used to determine the steady state kinetic parameters of WT and PRMT1 mutants (Osborne et al. 2007). Assays were performed in a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 15 μM [¹⁴C]-labeled SAM, and a varying concentration of AcH4-21 (0-1000 μM final). Reactions were pre-incubated at 37 °C for 10 min and WT PRMT1, or a PRMT1 mutant, was then added and the reaction was quenched after 15 min. For the assays varying SAM (0-39.7 μM final), the same reaction mixture was used except the concentration of AcH4-21 was held constant at 100 μM. Each assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within ≤
20%. The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq 1 or eq 2 if substrate inhibition was observed,

\[
\nu = \frac{V_{\text{max}}[S]}{(K_m + [S])} \\
\nu = \frac{V_{\text{max}}[S]}{(K_m + [S])(1+[S]/K_i)}
\]

(1), (2).

2.2.6 MALDI-MS Based Activity Assay

A previously described matrix assisted laser desorption/ionization (MALDI) MS based assay was used to determine the processivity of WT and select PRMT1 mutants (Osborne et al. 2007). Briefly, assays were performed in a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 500 μM SAM, and 20 μM AcH4-21. Reactions were then pre-incubated at 37 °C for 10 min. WT PRMT1 or a PRMT1 mutant was then added and the reaction was quenched with 3 μL of 50% TFA in ddH2O after the appropriate time period. Spectra were acquired on a Bruker Ultraflex II MALDI-TOF MS or an Applied Biosystems 4800 Plus MALDI TOF/TOF MS and analyzed using Flex Analysis software. The percent turnover was determined by dividing the intensity of the modified peptide by the sum of the intensities of the unmodified and modified substrates times 100%.

2.2.7 Chemical Analysis of Methylation Products

A reaction mixture of 10 μg of GST-GAR and 1.4 μM [3H]-SAM in 50 mM HEPES at pH 7.5 was incubated with 2 μg of WT PRMT1 or a PRMT1 mutant for 2 h at 37 °C. The products were then precipitated with an equal volume of 50% trichloroacetic acid, washed with acetone, and hydrolyzed for 20 h at 110 °C in 6 M HCl. The hydrolysate was dried and mixed with standards of ADMA, SDMA, and ω-MMA before being fractionated on a high-resolution cation exchange column as described (Fisk et al.
2009). One-tenth of the fractions were used for ninhydrin analysis of the standards, and nine-tenths were counted.

2.2.8 Partial Proteolysis

Partial proteolysis assays were performed in a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, and in the absence or presence of 0.5 mM SAH and 0.75 μg/mL subtilisin on ice. WT PRMT1, or a PRMT1 mutant, was then added and the reaction was quenched after 60 min with 5 mM phenylmethylsulfonyl fluoride. Protein fragments were separated by 12% SDS-PAGE and visualized with Coomassie Brilliant Blue.

2.2.9 pH Profile

The steady state kinetic parameters for the WT enzyme as well as the Y39F and H293A mutants were determined over a pH range of 6.0-9.25 using the gel-based activity assay described above. Assays were performed in a reaction mixture of 50 mM Bis-Tris (6.0-7.0), 50 mM HEPES (7.0-8.5), 50 mM Tricine (8.5-9.0), or CHES (8.75-9.25), 1 mM EDTA, 50 mM NaCl, and 0.5 mM dithiothreitol, with constant and varying concentrations of $[^{14}\text{C}]-\text{SAM}$ (0-41 μM final) and RGG3 (0-1000 μM final). Each assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within ≤20%. Note that, in order to make certain that the variances in kinetic parameters were not the result of a buffer effect; an overlapping buffer method was utilized. The kinetic parameters for the overlapping buffers were similar and thus the average was used. In addition, time course assays were performed at each pH to demonstrate that activity was not lost over time. The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq 3 or eq 4,
\[
y = \frac{((\text{Lim}_1 + \text{Lim}_2 \times 10^{(\text{pH}-\text{pKa}_1)})/(10^{(\text{pH}-\text{pKa}_1)}+1) - ((\text{Lim}_2 - \text{Lim}_3) \times 10^{(\text{pH}-\text{pKa}_2)})/(10^{(\text{pH}-\text{pKa}_2)}+1)))}{((\text{Lim}_1 + \text{Lim}_2 + 10^{(\text{pH}-\text{pKa}_1)})/(10^{(\text{pH}-\text{pKa}_1)}+1))}
\]  
(3),  

For eq 3 and eq 4, Lim 1 corresponds to the activity measured at low pH, Lim2 corresponds to the maximum activity measured at the optimum pH, and for eq 3, Lim3 is equal to the activity measured at high pH.

2.2.10 Solvent Isotope Effect

SIEs were investigated by determining the steady state kinetic parameters for the WT enzyme using the gel-based activity assay described above. The reaction mixture consisted of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 15 μM [\(^{14}\)C]-labeled SAM, and a varying concentration of AcH4-21 (0-1000 μM final) in >92% D\(_2\)O. The assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within ≤20%. The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq 1.

2.2.11 SAH Inhibition Studies

The inhibition constants for SAH were determined for the WT and mutant enzymes using the gel-based activity assay described above. The reaction mixture consisted of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 15 μM [\(^{14}\)C]-labeled SAM, 100 μM AcH4-21, and a varying concentration of SAH (0-500 μM final). The assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within ≤ 20%. The GraFit version 5.0.11 software
(Leatherbarrow 2004) was used to fit the data to a Dixon plot of $1/\upsilon$ versus SAH concentration. The $K_i$ was determined using eq 5,

$$\text{slope} = K_m/(V_{\text{max}} \cdot K_i \cdot [S]) \quad (5)$$

2.2.12 pKa calculations

The structure of PRMT1 (PDB ID: 1ORI) was rebuilt using Amber topology parameters and hydrogen atoms added to the structure. Atom partial charges and atomic radii were assigned based on Amber99 force-field using the program AMBER. pK$_a$ value were computed taking into account desolvation effects and intra-protein interactions, including the proximity of neighboring functional groups.

2.3 Results and Discussion

To begin to investigate the catalytic mechanism of PRMT1, site directed mutagenesis was used to probe the roles of Y39, R54, E100, S102, E144, E153, M155, and H293. The mutant enzymes were purified and characterized according to described procedures (Osborne et al. 2007). AcH4-21, a 21 amino acid peptide based on the N-terminus of histone H4, was used as a substrate for PRMT1 and mutants (Table 2.1). The kinetic parameters of each mutant were determined for the AcH4-21 peptide as well as SAM (Table 2.2 & 2.3). Note that similarly to other systems (Boehr et al. 2001 & Knuckley et al. 2007)$^2$, partial proteolysis studies were performed to ensure that the loss of activity associated with a particular mutation was not due to a gross structural perturbation (data not shown).
Table 2.1 Peptide Sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcH4-21</td>
<td>1-Ac-SGRGKGGKGLGKGGAKRHRKV</td>
</tr>
<tr>
<td>RGG3</td>
<td>GGRGGFGGRRGFGGRGGFG</td>
</tr>
</tbody>
</table>

Table 2.2 Kinetic parameters of PRMT1 mutants for the AcH4-21 peptide

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( K_m ) (µM)</th>
<th>Fold</th>
<th>( k_{cat} ) (min(^{-1}))</th>
<th>Fold</th>
<th>( k_{cat}/K_m ) (M(^{-1})·min(^{-1}))</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(^a)</td>
<td>1.1 ± 0.5</td>
<td>-----</td>
<td>4.6 x 10(^{-1}) ± 2 x 10(^{-2})</td>
<td>-----</td>
<td>4.1 x 10(^5)</td>
<td>-----</td>
</tr>
<tr>
<td>Y39F(^a)</td>
<td>2.0 ± 0.7</td>
<td>1.8</td>
<td>4.2 x 10(^{-2}) ± 2 x 10(^{-3})</td>
<td>11</td>
<td>2.1 x 10(^4)</td>
<td>20</td>
</tr>
<tr>
<td>R54K(^a)</td>
<td>0.8 ± 0.6</td>
<td>0.7</td>
<td>1.09 x 10(^{-2}) ± 4 x 10(^{-4})</td>
<td>42</td>
<td>1.3 x 10(^4)</td>
<td>32</td>
</tr>
<tr>
<td>R54A(^a)</td>
<td>5 ± 1</td>
<td>4.5</td>
<td>6.0 x 10(^{-2}) ± 3 x 10(^{-3})</td>
<td>7.7</td>
<td>1.3 x 10(^4)</td>
<td>32</td>
</tr>
<tr>
<td>E100D(^a)</td>
<td>5 ± 1</td>
<td>4.5</td>
<td>3.6 x 10(^{-1}) ± 1 x 10(^{-2})</td>
<td>1.3</td>
<td>7.6 x 10(^4)</td>
<td>5.4</td>
</tr>
<tr>
<td>E100Q(^a)</td>
<td>6 ± 2</td>
<td>5.5</td>
<td>2.5 x 10(^{-1}) ± 1 x 10(^{-2})</td>
<td>1.8</td>
<td>4.5 x 10(^4)</td>
<td>9.1</td>
</tr>
<tr>
<td>E100A(^a)</td>
<td>2.6 ± 0.9</td>
<td>2.4</td>
<td>1.85 x 10(^{-1}) ± 8 x 10(^{-3})</td>
<td>2.5</td>
<td>7.2 x 10(^4)</td>
<td>5.7</td>
</tr>
<tr>
<td>E144D(^a)</td>
<td>1.7 ± 0.4</td>
<td>1.5</td>
<td>2.2 x 10(^{-1}) ± 1 x 10(^{-2})</td>
<td>2.0</td>
<td>1.3 x 10(^5)</td>
<td>3.2</td>
</tr>
<tr>
<td>E144Q(^a)</td>
<td>2.0 ± 0.6</td>
<td>1.8</td>
<td>1.3 x 10(^{-1}) ± 4 x 10(^{-3})</td>
<td>3.5</td>
<td>6.7 x 10(^4)</td>
<td>6.1</td>
</tr>
<tr>
<td>E144A(^a)</td>
<td>0.3 ± 0.2</td>
<td>0.3</td>
<td>9.5 x 10(^{-3}) ± 2 x 10(^{-4})</td>
<td>50</td>
<td>3 x 10(^4)</td>
<td>14</td>
</tr>
<tr>
<td>E153D(^a)</td>
<td>3.1 ± 0.5</td>
<td>2.8</td>
<td>2.65 x 10(^{-2}) ± 5 x 10(^{-4})</td>
<td>17</td>
<td>8.6 x 10(^3)</td>
<td>50</td>
</tr>
<tr>
<td>E153Q(^a)</td>
<td>2.0 ± 0.8</td>
<td>1.8</td>
<td>7.7 x 10(^{-3}) ± 3 x 10(^{-4})</td>
<td>60</td>
<td>3.8 x 10(^3)</td>
<td>110</td>
</tr>
<tr>
<td>E153A(^a)</td>
<td>1.8 ± 0.6</td>
<td>1.6</td>
<td>3.8 x 10(^{-3}) ± 1 x 10(^{-4})</td>
<td>121</td>
<td>2.2 x 10(^3)</td>
<td>190</td>
</tr>
<tr>
<td>E144A/E153A(^a)</td>
<td>3.0 ± 0.7</td>
<td>2.7</td>
<td>1.32 x 10(^{-1}) ± 4 x 10(^{-3})</td>
<td>3.5</td>
<td>4.4 x 10(^4)</td>
<td>9.3</td>
</tr>
<tr>
<td>M155L(^a)</td>
<td>0.6 ± 0.4</td>
<td>0.5</td>
<td>1.73 x 10(^{-1}) ± 5 x 10(^{-3})</td>
<td>2.7</td>
<td>3 x 10(^5)</td>
<td>1.4</td>
</tr>
<tr>
<td>M155A(^b)</td>
<td>1.8 ± 0.5</td>
<td>1.6</td>
<td>4.6 x 10(^{-2}) ± 1 x 10(^{-3})</td>
<td>10</td>
<td>2.6 x 10(^4)</td>
<td>16</td>
</tr>
<tr>
<td>H293Q(^a)</td>
<td>2 ± 2</td>
<td>1.8</td>
<td>7.6 x 10(^{-3}) ± 6 x 10(^{-4})</td>
<td>61</td>
<td>3.7 x 10(^3)</td>
<td>110</td>
</tr>
<tr>
<td>H293A(^a)</td>
<td>11.1 ± 0.6</td>
<td>10</td>
<td>1.81 x 10(^{-2}) ± 2 x 10(^{-4})</td>
<td>25</td>
<td>1.6 x 10(^3)</td>
<td>256</td>
</tr>
</tbody>
</table>

\(^a\)[SAM] = 15 µM. \(^b\)[SAM] = 30 µM.
2.3.1 Mutagenesis Studies of SAM Binding Mutants

2.3.1.1 R54 Mutants

R54 hydrogen bonds and/or forms an electrostatic interaction with E144, one of the two key glutamate residues, and presumably orients this residue for the productive recognition of the substrate guanidinium. Additionally, both Nη₁ and Nη₂ of this residue interact with the carboxylate group on the methionine portion of SAM, which suggests

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M⁻¹·min⁻¹)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTᵃ</td>
<td>6 ± 1</td>
<td>-----</td>
<td>5.8 x 10⁻¹ ± 3 x 10⁻²</td>
<td>-----</td>
<td>1 x 10⁵</td>
<td>-----</td>
</tr>
<tr>
<td>Y39Fᵃ</td>
<td>14 ± 3</td>
<td>2.3</td>
<td>7.0 x 10⁻² ± 5 x 10⁻³</td>
<td>8.3</td>
<td>5.1 x 10³</td>
<td>20</td>
</tr>
<tr>
<td>R54Kᵃ</td>
<td>14 ± 1</td>
<td>2.3</td>
<td>3.3 x 10⁻² ± 1 x 10⁻³</td>
<td>18</td>
<td>2.4 x 10³</td>
<td>42</td>
</tr>
<tr>
<td>R54Aᵃ</td>
<td>10 ± 1</td>
<td>1.7</td>
<td>9.5 x 10⁻² ± 4 x 10⁻³</td>
<td>6.1</td>
<td>9.1 x 10³</td>
<td>11</td>
</tr>
<tr>
<td>E100Dᵃ</td>
<td>6 ± 2</td>
<td>1.0</td>
<td>4.8 x 10⁻¹ ± 5 x 10⁻²</td>
<td>1.2</td>
<td>7.8 x 10⁴</td>
<td>1.3</td>
</tr>
<tr>
<td>E100Qᵃ</td>
<td>7 ± 2</td>
<td>1.2</td>
<td>2.8 x 10⁻¹ ± 2 x 10⁻²</td>
<td>2.1</td>
<td>3.8 x 10⁴</td>
<td>2.6</td>
</tr>
<tr>
<td>E100Aᵃ</td>
<td>5 ± 2</td>
<td>0.8</td>
<td>1.6 x 10⁻¹ ± 2 x 10⁻²</td>
<td>3.6</td>
<td>3.1 x 10⁴</td>
<td>3.2</td>
</tr>
<tr>
<td>E144Dᵃ</td>
<td>4.0 ± 0.9</td>
<td>0.7</td>
<td>2.7 x 10⁻¹ ± 2 x 10⁻²</td>
<td>2.1</td>
<td>6.8 x 10⁴</td>
<td>1.5</td>
</tr>
<tr>
<td>E144Qᵃ</td>
<td>8.3 ± 0.9</td>
<td>1.4</td>
<td>1.60 x 10⁻¹ ± 7 x 10⁻³</td>
<td>3.6</td>
<td>1.9 x 10⁴</td>
<td>5.3</td>
</tr>
<tr>
<td>E144Aᵃ</td>
<td>3.1 ± 0.6</td>
<td>0.5</td>
<td>1.05 x 10⁻² ± 4 x 10⁻⁴</td>
<td>55</td>
<td>3.4 x 10⁴</td>
<td>29</td>
</tr>
<tr>
<td>E153Dᵃ</td>
<td>5 ± 1</td>
<td>0.8</td>
<td>2.8 x 10⁻² ± 2 x 10⁻³</td>
<td>21</td>
<td>5.8 x 10³</td>
<td>17</td>
</tr>
<tr>
<td>E153Qᵃ</td>
<td>17 ± 4</td>
<td>2.8</td>
<td>1.9 x 10⁻² ± 2 x 10⁻³</td>
<td>31</td>
<td>1.1 x 10³</td>
<td>91</td>
</tr>
<tr>
<td>E153Aᵃ</td>
<td>9 ± 3</td>
<td>1.5</td>
<td>4.1 x 10⁻³ ± 4 x 10⁻⁴</td>
<td>141</td>
<td>4 x 10²</td>
<td>250</td>
</tr>
<tr>
<td>E144A/E153Aᵃ</td>
<td>10 ± 2</td>
<td>1.7</td>
<td>1.06 x 10⁻¹ ± 6 x 10⁻³</td>
<td>5.5</td>
<td>1.1 x 10⁴</td>
<td>9.1</td>
</tr>
<tr>
<td>M155Lᵃ</td>
<td>10 ± 2</td>
<td>1.7</td>
<td>1.00 x 10⁻¹ ± 8 x 10⁻³</td>
<td>5.8</td>
<td>1 x 10⁴</td>
<td>10</td>
</tr>
<tr>
<td>M155Aᵃ</td>
<td>110 ± 21</td>
<td>18</td>
<td>2.9 x 10⁻¹ ± 4 x 10⁻²</td>
<td>2.0</td>
<td>2.8 x 10³</td>
<td>36</td>
</tr>
<tr>
<td>H293Qᵃ</td>
<td>17 ± 6</td>
<td>2.8</td>
<td>1.4 x 10⁻² ± 2 x 10⁻³</td>
<td>41</td>
<td>8 x 10²</td>
<td>125</td>
</tr>
<tr>
<td>H293Aᵃ</td>
<td>37 ± 2</td>
<td>6.2</td>
<td>5.6 x 10⁻² ± 1 x 10⁻³</td>
<td>10</td>
<td>2 x 10³</td>
<td>50</td>
</tr>
</tbody>
</table>

ᵃ[AcH4-21] = 100 µM.
that this residue could play a key role in SAM binding (Figure 2.2). Consistent with this prediction is the 42-fold decrease in the $k_{\text{cat}}/K_m$ value observed for SAM with the R54K mutant. However, the effect on $k_{\text{cat}}/K_m$ is primarily driven by a decrease in $k_{\text{cat}}$ and not $K_m$, suggesting that the lack of a more dramatic effect on the SAM $K_m$ reflects the multi-step nature of the reaction, where $K_d$ is not equal to $K_m$; $k_{\text{cat}}/K_m$ represents all steps up to and including the first irreversible step of the reaction. To evaluate whether this was indeed the case, we determined the dissociation constants, i.e., $K_i$, for SAH binding to both the WT and R54A enzymes; SAH was used for these experiments as a proxy for SAM to more accurately gauge the effects of a particular mutation on SAM binding. The results of these studies confirm that R54 is not critical for SAM binding, as evidenced by the fact that the $K_i$ for SAH is similar to that obtained for the WT enzyme (Table 2.4).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>Y39F</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>R54A</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>E100A</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>M155A</td>
<td>34 ± 6</td>
</tr>
</tbody>
</table>

With respect to the peptide substrate, large changes in both $k_{\text{cat(app)}}$ (42- and 7.7-fold) and $k_{\text{cat}}/K_m$ (32- and 32-fold) were observed for both the R54K and R54A mutants, respectively. Although these effects are at least partially related to our inability to completely saturate the enzyme with SAM in our radioactive methyltransferase assay, which is why the term $k_{\text{cat(app)}}$ is used, their magnitude, particularly on $k_{\text{cat(app)}}$, is consistent with a role for this residue in orienting the substrate guanidinium via E144 for nucleophilic attack on the methyl group of SAM. Consistent with this notion is the fact
that the $k_{\text{cat}}$ values obtained with SAM, where the peptide substrate is saturating, are decreased by a similar order of magnitude.

2.3.1.2 E100 Mutants

E100 hydrogen bonds to the ribose moiety of SAM and thus would be expected to play an important role in SAM binding. Three mutants, i.e., the E100D, E100Q, and E100A mutants, were made to confirm this hypothesis. With respect to the kinetic parameters determined for SAM, there is only a very small effect on $k_{\text{cat}}/K_m$. For example, the complete removal of the E100 carboxylate, as occurs in the E100A mutant, decreases $k_{\text{cat}}/K_m$ by only 3.2-fold. The $K_i$ for SAH with this mutant is similarly unaffected, quite clearly demonstrating that this residue is not important for SAM binding or catalysis. This result is especially surprising when one considers that the distances between the ribose hydroxyls and the $\alpha$-carboxylate of E100 in the PRMT1●SAH complex are only 2.6-2.7 Å, which are distances typically associated with relatively strong hydrogen bonds. Although the observed interactions may be an artifact of the crystallization conditions (the enzyme was crystallized at pH ~4.7, which would favor protonation of E100 and potentially promote hydrogen bond formation), similar distances and orientations are observed in the crystal structures of the PRMT3●SAH and CARM1●SAH complexes which were crystallized at pH 6.3 and pH 7, respectively (Zhang et al. 2000, Troffer-Charlier et al. 2007). Thus, such an explanation is intellectually unsatisfying. Nevertheless, these results are at least partially consistent with the fact that methylthioadenosine is a relatively poor PRMT1 inhibitor (Osborne et al. 2007).
With respect to the kinetic parameters determined for the AcH4-21 peptide, the effects, while still small, are significantly larger than those observed with SAM. For example, the fold decrease in \( k_{\text{cat(app)}} / K_m \) for the E100D, E100Q, and E100A mutants are 5.4-fold, 9.1-fold, and 5.7-fold, respectively. As the effects are largely driven by an increase in \( K_m \), these results suggest that an interplay exists between the binding of both substrates, or alternatively, a change in the kinetic mechanism.

### 2.3.1.3 M155 Mutants

To probe the importance of M155, the M155L and M155A mutants were generated. With respect to the peptide substrate, the kinetic parameters for the M155L mutant were similar to those obtained for the WT enzyme; the \( k_{\text{cat(app)}} \) and \( k_{\text{cat(app)}} / K_m \) values were only decreased by 2.7-fold and 1.4-fold, respectively. In contrast to these relatively minor effects, 5.8- and 10-fold decreases in \( k_{\text{cat}} \) and \( k_{\text{cat}} / K_m \) were observed when SAM was tested as the varied substrate. These effects either represent an inability to properly position the S-methyl group of SAM or, alternatively, an effect on SAM binding. Given that M155 forms the bottom of the adenine portion of the SAM binding pocket, these results are most consistent with the latter possibility because the structural differences between a leucine and a methionine would be expected to alter SAM binding. Similar effects on SAM binding are observed with the M155A mutant. For example, the \( K_m \) and \( k_{\text{cat}} / K_m \) values of the M155A mutant for SAM are increased by 18- and 36-fold, respectively. Confirming that this residue is important for SAM binding is the fact that the \( K_i \) for SAH is increased by 27-fold relative to WT. The large changes in \( K_i, K_m \) and \( k_{\text{cat}} / K_m \) likely reflect a loss of steric constraint within the SAM binding pocket, which disfavors the binding of SAM in an orientation that is productive for catalysis. With
respect to the peptide substrate, 10- and 16-fold reductions in $k_{\text{cat(app)}}$ and $k_{\text{cat(app)}}/K_m$ were observed. These effects are most likely due to our inability to completely saturate the enzyme with SAM in our radioactive methyltransferase assay.

### 2.3.1.4 Role of M155 in ADMA Formation

Given the postulated role of M155 in directing the formation of ADMA, as opposed to SDMA (Zhang et al. 2000 & Branscombe et al. 2001), we also investigated, in collaboration with Cecilia Zurita-Lopez and Steve Clarke (UCLA), the contribution of this residue to the regiospecific dimethylation of a substrate arginine residue. For these studies, the M155A mutant was utilized because we hypothesized that this mutation would relieve the steric constraint imposed by the methionine and thereby open up the pocket and allow for SDMA formation. To investigate this possibility, the M155A mutation, along with the WT control, was used to catalyze the $^3$H-SAM dependent methylation of GST-GAR, a fusion protein that links GST to the N-terminus of human fibrilarin (Figure 2.3). Subsequently, the reaction was hydrolyzed in 6 M HCl at 110°C, and the extent of $\omega$-MMA, SDMA, and ADMA formed was quantified by high resolution cation exchange chromatography. The results of these experiments indicate that the M155A mutant catalyzes the exclusive formation of $\omega$-MMA and ADMA. Thus, despite the fact that this mutation relieves the steric constraint thought to prevent SDMA formation, our results indicate M155 is not responsible for the formation of ADMA over SDMA.

Nevertheless, given that the mutation of this residue strongly impacts the kinetics of the PRMT1 catalyzed reaction, particularly with respect to the $K_m$ for SAM, we reasoned that it may play a role in the processivity of ADMA formation. To investigate this
possibility, a previously established MALDI-MS assay (Osborne et al. 2007) was utilized. Consistent with previous results with the WT enzyme, ω-MMA and ADMA containing peptides were initially produced in equimolar amounts, followed by a decrease in levels of ω-MMA (Figure 2.4A). As described previously, these results are characteristic of an enzyme that has the ability to rebind SAM and subsequently produce.

Figure 2.3 Amino acid analysis of PRMT1-depended methylation products. (A) WT PRMT1 produces a mixture of ω-MMA and ADMA as products of methylation. (B) The M155A mutant also produces a mixture of ω-MMA and ADMA as products of methylation. In each case, the position of the standards was determined by ninhydrin assay (dotted lines); the position of the \[^3\text{H}\text{-radiolabeled derivatives by counting (solid lines). The slightly earlier elution position of the }[^3\text{H}]\text{-ADMA and }[^3\text{H}]\text{-ω-MMA products compared to the standards is due to the mass and pKa differences of amino acids with tritium versus hydrogen-containing methyl groups (Fisk et al. 2009).}$$

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Figure 2.4 Processivity of WT PRMT1 and M155 mutants. (A) WT PRMT1 uses a partially processive mechanism to catalyze the formation of ADMA. (B) The M155L mutant uses a partially processive mechanism, however, formation of ADMA is slower than with WT PRMT1. (C) The M155A mutant uses a distributive mechanism to catalyze the formation of ADMA. The large percent turnover of \( \omega \)-MMA suggests that \( \omega \)-MMA is released prior to the rebinding in preparation for the second round of methylation.

ADMA before the first methylation product, \( \omega \)-MMA, is released (Osborne et al. 2007). Because ADMA formation is not obligatory, we have suggested that PRMT1 displays partial processivity. With respect to the M155L mutant, similar results were obtained, however, the level of ADMA formed is significantly lower at the early time points (Figure 2.4B). This result indicates that the M155L mutant is significantly less processive than the WT enzyme. For the M155A mutant, little to no ADMA is formed
until the 20 min time point (Figure 2.4C), which suggests that ω-MMA is released prior to the rebinding of SAM and that this mutant utilizes a distributive mechanism. Note that the concentration of SAM used in these assays was 500 µM; thus the loss of processivity is not due to a failure to saturate the enzyme with SAM. In total, these results are consistent with M155 playing a key role in SAM binding, as reduced affinity for SAM, as occurs with both the M155L and M155A mutants, would be expected to decrease the processivity of the enzyme because, for these mutants, the off rate for the monomethylated peptide is larger than the rate constant for SAM binding.

2.3.2 Mutagenesis Studies of Catalytic Mutants

2.3.2.1 E144 Mutants

E144 appears to orient Nη2 of the substrate guanidinium group to facilitate nucleophilic attack on the S-methyl group of SAM. In order to investigate this role, the E144D, E144Q, and E144A mutants were generated. The $k_{cat}/K_{m}$ values for the E144D mutant were decreased by 3.2-fold and 1.5-fold for AcH4-21 and SAM, respectively. The ability of aspartate to substitute for the glutamate indicates flexibility within the active site and reveals that positioning of this residue is not critical for catalysis. For the E144Q mutant, the effects on $k_{cat}/K_{m}$ are slightly larger, with these values down 6.1-fold and 5.3-fold for AcH4-21 and SAM, respectively. These data suggest that the charge of this residue is significantly less important than its ability to hydrogen bond with both the substrate guanidinium and R54, as opposed to modulating the nucleophilicity of that group. Note that the calculated $pK_a$ of the E144 carboxylate is ~2.7; thus this residue is likely deprotonated in the PRMT1 active site. Nevertheless, this residue is important for catalysis, as illustrated by the 14-fold and 29-fold decrease in the $k_{cat}/K_{m}$ values for
AcH4-21 and SAM for the E144A mutant. Note the effects on $k_{\text{cat}}/K_m$ are dominated by a decreased $k_{\text{cat}}$, suggesting that this residue is relatively unimportant for substrate binding. These results are consistent with previous findings from mutagenesis studies that only measured relative rates (Zhang et al. 2003). In total, the data suggest that the hydrogen bonding characteristics, and to a lesser extent, the charge of E144, are important for orienting the substrate guanidinium for nucleophilic attack on the S-methyl group of SAM.

2.3.2.2 E153 Mutants

The carboxylate group of E153 is thought to play a crucial role in catalysis through its electrostatic and hydrogen bonding interactions with $N_{\eta_1}$ and $N_\delta$ of the substrate guanidinium. To investigate the importance of both the size and charge of this residue the E153D, E153Q, and E153A mutants were made and the kinetic parameters determined. The results are consistent with this residue playing a key role in catalysis. For example, the $k_{\text{cat}}$ values for the E153D, E153Q, and E153A mutants are decreased by 21-, 31-, and 140-fold, respectively. Given that the effects on $k_{\text{cat}}/K_m$ mirror the effects on $k_{\text{cat}}$, these data indicate that both the charge and the position of E153 are more important for catalysis than substrate binding. These results are also consistent with previous findings from mutagenesis studies that only measured relative rates (Zhang et al. 2003). The fact that mutating E153 has a more dramatic effect than mutating E144 is consistent with the notion that this residue plays an important role in redistributing electron density within the guanidinium group to enhance its nucleophilicity.

2.3.3.3 E144A/E153A Mutant

Given the results obtained for the E144 and E153 single mutants, we expected
that the E144A/E153A double mutant would likely yield negligible, if any, activity. However, this was not the case, as $k_{cat}$ is decreased by only 5.5-fold and the $k_{cat}/K_m$ values are only decreased by 9.3- and 9.1-fold for AcH4-21 and SAM, respectively. The more significant effect on the single mutants is most easily explained by the formation of a strong salt bridge to the remaining glutamate. Such an interaction would be expected to decrease activity by altering both the nucleophilicity of the guanidinium and its position such that methyl transfer is suboptimal. The fact that the double mutant retains considerable activity is more difficult to rationalize. However, it is possible that the removal of the two glutamate residues increases the hydrophobicity of the active site, which would be expected to depress the $pK_a$ of the guanidinium, and thereby increase its nucleophilicity. Although we cannot rule out such a possibility, the active site possesses a number of other hydrophilic residues in close proximity (e.g., Y39, R54, and H293) that should minimize any change in hydrophobicity. An alternative explanation that is equally supported by the data involves the PRMT1 catalyzed reaction being primarily driven by bringing the substrate guanidinium into close proximity to the $S$-methyl group of SAM.

**2.3.3.4 H293 Mutants**

H293 has been thought to play the role of a general base by accepting a proton from $N_\eta_2$, however, this residue is >6 Å from the approximate position of the substrate guanidinium, a distance that is too far to directly remove the proton. Although a water-mediated proton transfer mechanism is possible, the high basicity of the substrate guanidinium makes this mechanism intellectually unsatisfying and we have suggested (see above) that proton loss could occur prior to, concomitant with, or even after methyl
transfer, and that H293 may not act as a general base. In order to probe this hypothesis, the H293Q and H293A mutants were generated. For the H293Q mutant, the $k_{\text{cat}}$ is decreased by 41-fold, whereas negligible changes in the $K_m$ values were observed, indicating that the 110- and 125-fold decreases in the $k_{\text{cat}}/K_m$ values for AcH4-21 and SAM are driven by $k_{\text{cat}}$. For the H293A mutant, the effects on $k_{\text{cat}}/K_m$ are similar in magnitude, with the $k_{\text{cat}}/K_m$ values for AcH4-21 and SAM decreased by 256- and 50-fold, respectively. Although these results indicate that H293 plays a critical role in catalysis, and could be interpreted as being consistent with a role for H293 as the general base, alternative explanations are also possible. For example, in the structure of the PRMT1•SAH complex, H293 appears to form a salt bridge with D51, a conserved residue that is present on αY. Given the short distance between the side chains of H293 and D51 (i.e., 2.6 Å), this interaction likely plays a critical role in forming the two helix boundary that separates the SAM and peptide binding portions of the active site (Figure 2.5). As such, one would expect that disruption of this interaction would lead to decreased activity via the inability to properly form the substrate and cofactor binding pockets. This is especially likely when one considers that Y39, H45, M48, and R54 are present on helix αY and αZ and likely play key roles in both PRMT1 catalysis (e.g., Y39 and R54) and forming the active site cleft (e.g., H45 and M48). Consistent with this possible role for H293 is the fact that similar effects on $k_{\text{cat}}/K_m$ are observed for both SAM and the AcH4-21 peptide. This is the case because either the alanine or glutamine mutations would not be expected to affect SAM binding, only $k_{\text{cat}}$. Given that R54 is also present on helix αY some of the effects of mutating this residue may also be due to the destabilizing the formation of the PRMT1 substrate and cofactor binding pockets.
Figure 2.5 D51 and H293 interaction. The kinetic effects of the H293 mutations can be explained by the fact that a salt bridge likely forms between D51 and H293, which are only separated by 2.6 Å. A disruption of this interaction could possibly prevent the proper formation of the binding pockets. This figure was prepared with UCSF Chimera using the coordinates for PRMT1 (PDB 1OR1).

### 2.3.3.5 Y39 Mutant

In CARM1, Y154 appears to be important for both cofactor binding and orienting E267 (Y154 and E267 correspond to Y39 and E153 in PRMT1) (Yue et al. 2007, Troffer-Charlier et al. 2007, Feng et al. 2009). Although the Y154F mutant appears to abolish CARM1 activity, this was a single point assay and only relative rates were measured (Feng et al. 2009). Thus, to establish the role of the corresponding residue in PRMT1, the Y39F mutant was generated and the kinetic parameters determined. Although this mutation has only small, ~2-fold effects on the $K_m$ values for AcH4-21 and SAM, the $K_i$ for SAH is increased by ~20-fold (Table 2.4), thereby confirming that this residue is important for cofactor binding; the lack of a more dramatic effect on the SAM $K_m$ reflects the multi-step nature of the reaction, where $K_d$ is not equal to $K_m$. The importance of this
residue was further illustrated by the 20-fold decrease in the $k_{\text{cat}}/K_m$ value observed for AcH4-21 and the 20-fold decrease seen with SAM. The position of this residue within the PRMT1 active site and the fact that $k_{\text{cat}}$ is also decreased by 8.3 fold suggest that this residue also plays an important role in rate enhancement; the specific role of this residue in catalysis is described below (see pH studies).

2.4 pH Studies

In order to further increase our understanding of PRMT1 catalysis, pH rate profiles were generated for the WT enzyme by determining $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values for both SAM and the RGG3 peptide over a pH range of 6.0-9.25. The RGG3 peptide (Table 2.1) was used in place of the AcH4-21 peptide to simplify the interpretation of the pH rate profiles. This is the case because this peptide has comparable kinetic parameters to the AcH4-21 peptide and, more importantly, because, with the exception of the N-terminus (pKa ~ 8.0), this peptide lacks residues that typically ionize within the pH range under study. Note that at all pH values enzyme activity was linear with respect to time, indicating that the loss of activity at the pH extremes was not due to a non-specific effect on enzyme structure. Also note that $k_{\text{cat}}/K_m$ is the apparent second order rate constant for the reaction of free substrate and free enzyme (or when one substrate (A) is saturating, the EA complex), and therefore the pH-dependence of $k_{\text{cat}}/K_m$ monitors the ionization state of these entities. In contrast, effects on $k_{\text{cat}}$ are interpreted as being due to the presence of important ionizable groups in the enzyme-substrate complex.

With SAM as the varied substrate, the plot of log $k_{\text{cat}}/K_m$ versus pH is bell-shaped and is consistent with the presence of two ionizable groups that are important for substrate capture; pK$_a$ values of 6.2 ± 0.3 and 10.5 ± 0.4 were assigned to the ascending
and descending limbs, respectively (Figure 2.6A). With respect to \( k_{\text{cat}} \), the rate of the reaction increases with increasing pH until a limiting value is reached (Figure 2.6B). Fitting the data to eq 4 yields a pK\(_a\) value of 5.1 ± 0.8. Although it is difficult to definitively assign an observed pK\(_a\) to a particular residue, or functional group on a substrate, the structures of PRMT1, SAM, and the RGG3 peptide suggest several possible residues/functional groups whose ionization could alter substrate capture or \( k_{\text{cat}} \). With respect to the ascending limb, protonation of the SAM carboxylate (pK\(_a\) ~ 2 in solution), D51, E100, E144, and E153 could explain the loss in activity as the pH decreases. However, most of these groups are readily ruled out. For example, the pK\(_a\) of the SAM carboxylate is significantly lower than the pK\(_a\) for the ascending limb of the \( k_{\text{cat}}/K_m \) versus pH-rate profile. Consistent with this notion is the fact that the R54A mutation does not affect the \( K_i \) for SAM. Although protonation of D51, which is the residue that interacts with H293, could also explain the loss of activity at low pH, the fact that the pH rate profiles obtained for the H293A mutant show a similar loss of activity at low pH (vide infra) argues against this possibility. Protonation of E100 could also potentially explain the loss of activity at low pH. However, the fact that little to no effect on the kinetic parameters were observed when this residue was mutated to alanine makes this suggestion unlikely. Of the remaining two residues, \textit{i.e.}, E144 and E153, the residue most likely responsible for the loss of activity at low pH is E153. We surmise that this is the case because the E144Q mutant retains considerable activity; thus, the ionization of this residue would also be expected to minimally impact the reaction rate. In contrast, the activity of the E153Q mutant is significantly decreased and similar to that obtained for the E153A mutant, thereby suggesting that protonation of the E153 carboxylate would
Figure 2.6. pH profiles of WT PRMT1 with SAM. (A) The log $k_{\text{cat}}/K_m$ versus pH plot is used to determine the pK$_a$ of ionizable groups on the enzyme or substrate. (B) The log $k_{\text{cat}}$ versus pH plot is used to determine the pK$_a$ of ionizable groups in the ES complex.
have a profound negative impact on rate acceleration. Also consistent with the ionization of this residue corresponding to the ascending limb is the fact that the pK\textsubscript{a} of a glutamate residue in solution is typically in the range of 4-5. In total, these data suggest that E153 must be deprotonated for optimal PRMT1 activity.

With respect to the descending limb, deprotonation of the amino group on SAM (pK\textsubscript{a} \approx 9.5 in solution), the N-terminal amino group on the RGG3 peptide (pK\textsubscript{a} \approx 8 in solution), Y39, and H293 could explain the loss in activity as the pH increases. Given the similarities in the pK\textsubscript{a} values of these functional groups, it is more difficult to definitively assign the pK\textsubscript{a} of the descending limb. Nevertheless, the fact that the pK\textsubscript{a} of the N-terminal amine on the RGG3 peptide is significantly lower than the pK\textsubscript{a} of the descending limb argues against the notion that the ionization of this group is responsible for the decreased activity at high pH. Note also that the pK\textsubscript{a} of an arginine residue is 12.5 and is significantly higher than the pK\textsubscript{a} observed for the descending limb, arguing against the idea that ionization of these residues in the RGG3 peptide is responsible for the loss in activity. The fact that the concentration of the RGG3 peptide is fixed in these experiments further argues against these possibilities because, here, \( k_{\text{cat}}/K_m \) is the second order rate constant for the reaction of SAM with the E•RGG3 complex, and thus, \( k_{\text{cat}}/K_m \) monitors the ionization state of these entities and not the RGG3 peptide. The fact that the calculated pK\textsubscript{a} of H293 (7.9) is significantly lower than the pK\textsubscript{a} observed for the descending limb suggests that this residue is also not responsible for the loss in activity at high pH. Of the two remaining functional groups/residues, \textit{i.e.}, the amino group on SAM and the Y39 phenol, deprotonation of either one could be responsible for the loss in
activity at high pH, as the pK$_a$ values of these groups (*i.e.*, 9.5 and 10.5, respectively) are similar to that obtained for the descending limb.

When the RGG3 peptide is used as the varied substrate, the plot of log $k_{cat}/K_m$ versus pH is also bell-shaped with an ascending limb pK$_a$ of 5.2 ± 0.2 and a descending limb pK$_a$ of 10.0 ± 0.3 (Figure 2.7A). For the same reasons described above, these pK$_a$ values likely correspond to the protonation states of E153 and Y39, respectively. Note that the assignment of the descending limb to the amino group on SAM can be at least partially ruled out because the concentration of SAM is fixed in these experiments; thus, $k_{cat}/K_m$ is the second order rate constant for the reaction of the RGG3 peptide with the E•SAM complex, and, as such $k_{cat}/K_m$ monitors the ionization state of these entities, and not SAM. Interestingly, and in contrast to the data presented for SAM, the plot of the log $k_{cat}$ values versus pH is relatively flat, thereby indicating that when the peptide is the varied substrate the turnover number is not influenced by pH (Figure 2.7B). This difference most likely reflects a change in the rate determining step for the reaction. Given that PRMT1 methylates its substrates in a partially processive fashion, this data is most consistent with product release being rate limiting when the peptide is the varied substrate, as opposed to the case when the concentration of SAM is varied and chemistry, a conformational change, or SAM binding is potentially rate-limiting.

pH-rate profiles were also generated for the H293A and Y39F mutants using the RGG3 peptide as the varied substrate (Figure 2.7). Note that we focused on these mutants because of their putative roles as the general base and because the tyrosine residue possesses an ionizable group that potentially corresponds to the basic limb of the WT pH-rate profile. Also note that we only generated profiles for the RGG3 peptide
Figure 2.7. pH profiles of WT PRMT1 and mutants with the RGG3 peptide. (A) The log $k_{\text{cat}}/K_m$ versus pH plot is used to determine the $pK_a$ of ionizable groups on the enzyme or substrate. (B) The log $k_{\text{cat}}$ versus pH plot is used to determine the $pK_a$ of ionizable groups in the ES complex.
because we envisioned that this data would provide greater insights into the factors that are important for promoting methyl transfer to the peptide substrate. For the H293A and Y39F mutants, the plots of log $k_{\text{cat}}$ versus pH are sigmoidal, with the rates increasing as a function of pH to a limiting value (Figure 2.7B). Fitting the data to eq 4 identified $pK_a$ values of 4.8 ± 0.4 and 5.1 ± 0.8 for the H293A and Y39F mutants, respectively. Interestingly, these data differ substantially from that obtained with the WT enzyme, where changes in pH did not affect $k_{\text{cat}}$. This difference likely reflects a change in the rate limiting step. Consistent with this possibility is the fact that the mutation of either residue decreases $k_{\text{cat}}$ by ≥11-fold; thus, chemistry is potentially rate limiting for both the H293A and Y39F mutants. In total, these data indicate that both residues play an important role in rate enhancement.

With respect to $k_{\text{cat}}/K_m$, the plots of log $k_{\text{cat}}/K_m$ versus pH for both the WT and H293A are similarly bell-shaped, and the $pK_a$ values obtained with the H293A mutant (i.e., 5.2 ± 1.6 and 10.1 ± 0.9) are nearly identical to those obtained for the WT enzyme (Figure 2.7A). Conversely, only the ascending limb is evident in the Y39F $k_{\text{cat}}/K_m$ versus pH plot, which suggests that the phenolic side chain of this residue corresponds to the descending limb of the $k_{\text{cat}}/K_m$ versus pH rate profile. These results suggest that Y39 must be protonated for optimal activity and that deprotonation of the phenol leads to a decrease in activity either by the loss of a key hydrogen bond between Y39 and E153 or electrostatic repulsion between these two residues that leads to a decrease in enzyme activity. In contrast, it is interesting to note that the lack of an effect of mutating H293 on the shape of the pH-rate profiles, suggests that the general acid/base properties of this
residue are not important for rate enhancement. As such, these data suggest that H293 does not act as the general base that deprotonates the substrate guanidinium.

2.4 Solvent Isotope Effects

To further probe the catalytic mechanism of PRMT1, we also determined the steady state kinetic parameters for the PRMT1 catalyzed reaction in D$_2$O, using the AcH4-21 peptide as the varied substrate. For these experiments, the rates of the reaction were measured in ≥92% D$_2$O and compared to those obtained in H$_2$O at the corresponding pL. For the WT enzyme, a small inverse SIE (0.9) is apparent on $k_{\text{cat}}/K_m$ when the peptide is the varied substrate (Table 2.5). Although this result could be interpreted as being consistent with general base catalysis being unimportant for rate enhancement (a normal SIE would be expected if general base catalysis plays a prominent role in rate enhancement), the fact that chemistry is unlikely to be rate limiting for the peptide substrate with the wild type enzyme (see above), could suggest that the lack of an effect is due to the fact that product release is insensitive to the identity of the solvent. Given this possibility, we also determined SIEs for the Y39F, R54A, E144A/E153A, and H293A mutants, because the large decreases in $k_{\text{cat}}$ (~5- to 20-fold) suggest that chemistry is rate limiting for these enzymes. To help to confirm this

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Figure 2.8 Processivity of WT PRMT1 and mutants. (A) WT PRMT1 uses a partially processive mechanism to catalyze the formation of ADMA. (B-D) The Y39F, R54A, and H293A mutants use a distributive mechanism to catalyze the formation of ADMA in which ω-MMA is released prior to rebinding in preparation for the second round of methylation. (E) The E144A/E153A mutant uses a partially processive mechanism, however, the formation of ADMA is slower than WT.
possibility, we examined the processivity of the above described mutant enzymes. The results of these studies (Figure 2.8) indicated that the Y39F, R54A, E144A/E153A, and H293A mutants do not methylate the AcH4-21 peptide in a partially processive fashion, further suggesting that chemistry is rate-limiting for at least a subset of these mutant enzymes. Note that for the majority of the mutants examined, relatively small inverse SIEs ($\text{SIE}_{k_{\text{cat}}}$ and $\text{SIE}_{k_{\text{cat}}/K_m} \sim 0.6$ to 0.9) were observed on both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ (Table 2.5). The two exceptions are the cases of the R54A and H293A mutants. For the R54A mutant, the $\text{SIE}_{k_{\text{cat}}}$ is small and normal ($\text{SIE}_{k_{\text{cat}}} = 1.2$), whereas for the H293A mutant the SIE on both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ is large and inverse ($\text{SIE}_{k_{\text{cat}}}$ and $\text{SIE}_{k_{\text{cat}}/K_m} = 0.1$). Note that these experiments were performed in parallel and that representative data from one of at least two independent experiments is reported in Table 2.5.

Inverse SIEs are often attributed to medium effects, the dissociation of a metal chelated water, viscosity effects, or effects on thiol ionization (Karsten et al. 1995). Although medium effects are difficult to exclude, they are typically small and normally ignored (Karsten et al. 1995). Additionally, the lack of a requirement for metal ions or the presence of a thiol within the active site of PRMT1 suggests that the observed inverse SIE is not due to either of these possibilities. To control for the effect of the increased viscosity of D$_2$O, the kinetic parameters were determined in the presence of 10% glycerol, a concentration of glycerol that closely mimics the viscosity of D$_2$O (Table 2.5). The results of these solvent viscosity experiments (SVEs) indicate that, with the exception of the R54A and H293A mutants, the $k_{\text{cat}}$ values for the WT and mutant enzymes were accelerated in the presence of 10% glycerol, suggesting that for these mutants the inverse SIE on $k_{\text{cat}}$ can be attributed to a viscosity effect. With respect to
inverse SVEs were detected for only the WT and E144A/E153A double mutant, suggesting again that for these mutants the inverse SIE on \( k_{\text{cat}}/K_m \) can be attributed to a viscosity effect. However, normal SVEs were observed for the Y39F, R54A, and H293A mutants, indicating that the inverse SIEs are due to a direct effect of D\(_2\)O on the PRMT1 catalyzed reaction. Although it is difficult to speculate on the molecular basis for the inverse SIEs, there are at least two possible explanations. First, given that deuterium atoms are known to form stronger hydrogen bonds, D\(_2\)O may stabilize the structure of PRMT1 and thereby enhance the rate of the reaction. This may be particularly true for the H293A mutant, where a very large, and likely unprecedented, inverse SIE was observed (SIE = 0.1). As mentioned previously, the disruption of the salt bridge between H293 and D51, which would be caused by the alanine mutation, likely prevents the proper formation of the substrate and cofactor binding pockets. However, when the H293A mutant is assayed in D\(_2\)O, this solvent stabilizes the structure of PRMT1 and compensates for the loss of the interaction between H293 and D51. As \( k_{\text{cat}}/K_m \) reports on all steps up to including the first irreversible step of the reaction, which for PRMT1 is likely methyl transfer, the observed inverse SIEs for the mutant enzymes may, alternatively, be reporting on the formation of the dication intermediate because rehybridization of the \( \omega \)-nitrogen from \( sp^2 \) to \( sp^3 \) would be expected to yield an inverse isotope effect. Regardless of the nature of the inverse SIE, the lack of a normal SIE for WT PRMT1, and all of the catalytically impaired mutants, suggests that general base catalysis is unimportant for the PRMT1 catalyzed reaction, and, more specifically, suggest that H293 does not act as a general base.
2.4 Conclusions

PRMT1 activity impacts a number of important cell signaling pathways (e.g., gene transcription), and is dysregulated in a number of human diseases including heart disease and cancer. Given its role in these diseases, PRMT1 represents a novel therapeutic target, and, as such, we have been focused on developing inhibitors targeting this isozyme (Osborne et al. 2007, Osborne et al. 2008, Obianyo et al. 2010, Bicker et al. 2010). In order to gain insights that could guide the design of inhibitors with increased potency and selectivity, we used a combination of site directed mutagenesis, pH rate profiles, and SIEs to begin to characterize the catalytic mechanism of PRMT1. For the mutagenesis studies, we focused our efforts on examining the contribution of eight residues lining the active site pocket of PRMT1, including Y39, R54, E100, S102, E144, E153, M155, and H293, which, based on structures of PRMT family members, have been hypothesized to be important for SAM binding (i.e., Y39, R54, E100), the regiospecific generation of ADMA (i.e., M155), the regulation of CARM1 (i.e., S102 and Y39), general base catalysis (i.e., H293), and modulating the nucleophilicity of the substrate guanidinium (i.e., E144 and E153).

The results of the mutagenesis studies indicate that while R54 and E100 form hydrogen bonds and electrostatic interactions with the SAM carboxylate and the ribose moiety, respectively, neither residue is important for SAM binding. However, the fact that the R54 mutations negatively impact the kinetic parameters obtained for the peptide substrate, helps to confirm that the observed hydrogen bond or electrostatic interaction between the R54 guanidinium and the E144 carboxylate is important for rate enhancement; this interaction likely orients E144 such that it can properly position Nη2
for nucleophilic attack on the S-methyl group of SAM. In contrast to R54 and E100, M155 is important for SAM binding, as evidenced by the 26-fold increase in the $K_i$ for SAH when this residue is mutated to alanine. The $k_{cat}/K_m$ obtained for SAM is similarly affected. As M155 forms the bottom of the adenine portion of the SAM binding pocket, the loss of hydrophobic interactions between M155 and the adenine ring, as occurs both the M155L and M155A mutants, likely results in both a loss of affinity and an inability to properly position the cofactor for methyl transfer. M155 is also important for the processivity, but not the regiospecificity of the PRMT1 catalyzed reaction, i.e., this residue does not direct the formation of ADMA over SDMA, as has previously been suggested.

E144 and E153 have previously been suggested to orient the substrate guanidinium and modulate its nucleophilicity to promote methyl transfer (Zhang et al. 2000). Consistent with previous mutagenesis studies that only measured relative rates (Zhang et al. 2000), our results indicate that both residues are important for PRMT1 catalysis. Interestingly, however, the charge and position of E144 appear to be relatively unimportant for rate enhancement as both the E144D and E144Q mutants retain considerable activity. These results suggest that the hydrogen bond between E144 and the substrate guanidinium is most important, and that this interaction likely orients $N_\eta_2$ for nucleophilic attack on the S-methyl group of SAM. In contrast, both the charge and position of E153 are important for rate enhancement, and the results are consistent with the previous suggestion that this residue modulates the nucleophilicity of the guanidinium group by redistributing electron density toward $N_\eta_1$ and $N_\delta$. 
With respect to Y39, in addition to being important for SAM binding (due to its position at the top of the SAM binding pocket), this residue appears to be important for rate enhancement. This is apparent from the 20-fold decrease in $k_{\text{cat}}/K_m$ when the peptide is the varied substrate. This result indicates that the phenolic hydroxyl group enhances the rate of catalysis. Based on structures of PRMT1 family members, this residue likely hydrogen bonds to E153, and this interaction is important for positioning the E153 carboxylate such that it can modulate the nucleophilicity of the substrate guanidinium. This hypothesis is supported by the fact that PRMT1 loses activity at high pH, where the deprotonated form of Y39 would be expected to predominate and the resultant electrostatic repulsions between this residue and the E153 carboxylate would lead to a loss in activity. This latter observation is further supported by the loss of the high pK$_a$ when this residue is mutated to phenylalanine.

The results obtained for the H293A mutant are particularly interesting. Although this residue has been suggested to act as general base to deprotonate the substrate guanidinium and thereby enhance the nucleophilicity of this group, our results do not support such a hypothesis despite the fact that mutation of this residue decreases $k_{\text{cat}}/K_m$ by $\geq 50$-fold. This is the case because effects are observed on both the kinetic parameters determined for SAM and the peptide substrate. Additionally, the pH rate profiles obtained for the H293A mutant are similar to those obtained for the WT enzyme, which indicates that the ionization of this residue does not contribute to either the rate limiting step of the reaction or substrate capture. Although the contribution of this residue to rate enhancement may not be apparent in the pH rate profiles, because this residue is unimportant for substrate capture or the rate-limiting step of the reaction, we deem this
possibility unlikely and suggest that the decreased activity observed when this residue is mutated is due to the loss of a critical salt bridge between this residue and D51. The loss of this salt bridge would be expected to destabilize the two N-terminal helices and impact cofactor and peptide binding, both of which occur when this residue is mutated. The SIE data also do not support a role for this residue as a general base, as a large and inverse SIE was observed for the H293A mutant. Further support for the idea that H293 is not a general base comes from the structure of PRMT1, which shows that this residue is ≥6 Å distal from the approximate site of the substrate guanidinium, a distance that is too great for this residue to play such a role.

In total, the data described above support a mechanism in which SAM and a protein, or peptide, substrate bind to the enzyme in a random fashion to form a ternary complex (Figure 2.2). E153 then likely redistributes the electron density towards either Nη1 or Nδ, or even both, which enhances the nucleophilicity of Nη2. The methyl group of SAM is then transferred to the protonated guanidinium of the substrate arginine to form a dication intermediate. Although such an intermediate is to our knowledge unprecedented, dianionic carboxylate intermediates have been proposed for several enzymes. Rehybridization of the guanidinium destabilizes the dication intermediate, thereby facilitating the loss of the extra proton to water or an unknown general base. While E144 could serve such a role, this seems unlikely when one considers that the effect of mutating this residue to glutamine has only a small impact on \( k_{\text{cat}}/K_m \). Further support for the notion that methyl transfer precedes proton transfer comes from the lack of a normal SIE on both the wild type enzyme as well as several catalytically impaired mutants; a normal SIE would be expected if proton abstraction was rate-limiting. Given
that Hedstrom and colleagues (Guillen Schlippe et al. 2005) have noted that in several enzymes arginine residues can act as general bases and that the $pK_a$ of those residues is potentially modulated by slight structural perturbations to the normally planar guanidinium, we cannot completely rule out the possibility that E144 and E153 depress the $pK_a$ of the substrate arginine and thereby enhance its nucleophilicity, via such a mechanism. However, it is difficult to rationalize such a mechanism with our findings that the E144A/E153A double mutant possesses considerably more activity than either of the single mutations alone. Thus, we favor the mechanism proposed above where the methyl group is transferred to the protonated guanidinium. In summary, our results suggest that the PRMT1-catalyzed reaction is primarily driven by bringing the substrate and cofactor into the proximity of each other and that the prior deprotonation of the substrate guanidinium is not required for methyl transfer.
CHAPTER 3

Regulation of PRMT1 by Post-translational Modifications

3.1 Introduction

Post-translational modifications play a pivotal role in the cell by adding an additional level of functional regulation. For example, the methylation of arginine residues within proteins, which is catalyzed by the PRMT family of enzymes, has been found to affect gene transcription, RNA splicing, signal transduction, and cell proliferation (Bedford et al. 2005, Bedford et al. 2007, Bedford et al. 2009, Wolf et al. 2009, and Di Lorenzo et al. 2011). Due to its numerous and various roles in cellular processes, it is only logical that arginine methylation itself would be regulated. One such method of regulation of the PRMT family is that of PTMs.

In fact, the activity of both CARM1 and PRMT5 has been found to be regulated by phosphorylation. In CARM1, three different serine residues, i.e., S217, S228, and S448, have been demonstrated to be phosphorylated in vivo (Feng et al. 2009, Higashimoto et al. 2007, Carascossa et al. 2010). S217 was identified by mass spectrometry on immunoprecipitated CARM1 from MCF-7 cells and this residue is conserved amongst Type I PRMTs. Phosphorylation of this residue diminishes the methyltransferase activity of the enzyme in vitro and abrogates its coactivator function in vivo. Additionally, this PTM causes the mainly nuclear enzyme to localize in the
cytoplasm. Based on the structure of CARM1, this change in activity is presumably due to the addition of a phosphate group to the side chain hydroxyl of S217, disrupting the hydrogen bond to the backbone carbonyl of Y154 (Feng et al. 2009). As a result, the enzyme cannot bind SAM. Additionally, this PTM could affect the position of Y154 (Y39 in PRMT1) (Feng et al. 2009), which has been suggested to interact with the ribose and sulfur atom of SAM, as well as with E267, a residue important for catalysis (Yue et al. 2007, Troffer-Charlier et al. 2007, & Rust et al. 2011). S228, which is conserved among different species (but not among different PRMT family members), was also identified by mass spectrometry on immunoprecipitated CARM1 from HELA and MCF-7 cells. Mutation of S228 to glutamate inhibited methyltransferase activity in vitro and abrogated its coactivator function in vivo. These results are likely due to the disruption of dimer formation (Higashimoto et al. 2007). Finally, S448 was identified from MCF-7 cells using an antibody against a PKA substrate consensus motif and verified by site-directed mutagenesis. Interestingly, unlike the previous two sites that were involved in regulating methyltransferase activity, phosphorylation of this residue mediates the interaction between CARM1 and apo-ERα and plays a role in ligand independent activation of the receptor by cAMP (Carascossa et al. 2010).

PRMT5 is also phosphorylated in vivo at three tyrosine residues (i.e., Y297, Y304, and Y307) that are conserved amongst species. Phosphorylation greatly decreases the methyltransferase activity of the enzyme in vitro against histones H2A and H4 and inhibits its association in vivo with MEP50, a known interacting partner that is critical for activity. Intriguingly, the responsible kinases are constitutively active mutants of Janus
kinase 2 (JAK2), i.e., JAK2V617F and JAKK539L, and the phosphorylation of PRMT5 by these mutants likely promotes myeloproliferative neoplasms (Liu et al. 2011).

In addition to phosphorylation, automethylation has been detected in select PRMT family members (i.e., CARM1 (Kuhn et al. 2011), PRMT6 (Frankel et al. 2002), and PRMT8 (Sayegh et al. 2007)). Although the in vitro methyltransferase activity of CARM1 is not affected by automethylation of R551 in its C-terminal domain (CTD), as determined using an arginine to lysine mutant, this modification appears to down regulate its roles in mRNA splicing and transcriptional activation (Kuhn et al. 2011). PRMT8 is monomethylated at R58 and dimethylated at R73 of its N-terminal tail. Interestingly, full length PRMT8 has very limited activity; however, a truncation mutant lacking the first 60 residues is significantly more active. The effect of automethylation on this isozyme remains to be determined, but it is speculated that it might alter substrate recognition (Sayegh et al. 2007). Finally, the effect of automethylation on PRMT6 is unknown at this time (Frankel et al. 2002).

As mentioned previously, our research is concentrated on PRMT1 because it is the dominant Type I PRMT and plays a role in a variety of diseases (e.g., cancer, heart disease, and ALS). We have been focused thus far on the kinetic and catalytic mechanisms of the enzyme, as well as the development of potent inhibitors, while other groups have mainly focused on determining the functional roles of PRMT1. The mechanisms (i.e., PTMs and protein-protein interactions) by which this otherwise constitutively active enzyme is regulated, however, have yet to be fully investigated. In fact, knowledge of the regulation of PRMT1 by post-translational modifications is extremely limited. PhosphoSitePlus® (www.phosphosite.org) (Hornbeck et al. 2012), a
curated source for experimentally determined PTMs, lists phosphorylation, ubiquitylation, and acetylation as observed modifications to a variety of PRMT1 residues (Table 3.1). Interestingly, the data for most, if not all, of the discovered PTMs arose from cancer cell lines such as K-562, AMO-1, and KMS-27 (Hornbeck et al. 2012), which would argue for a role for PRMT1 in disease. These results, however, are only based on high throughput mass spectrometry data, with no site specific methods such as the use of site-directed mutagenesis or specific antibodies, to verify the existence and/or importance of this modification.

**Table 3.1 Observed modifications of PRMT1**

<table>
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<th>Modification</th>
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<td>EDMTSKDyyFDSYAH</td>
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<tr>
<td>1</td>
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<td>Y35</td>
<td>EDMTSKDyyFDSYAH</td>
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<td>K113</td>
<td>DYAVKIVkANkLDHV</td>
</tr>
<tr>
<td>10</td>
<td>Ubiquitination</td>
<td>K116</td>
<td>DYAVKIVkANkLDHV</td>
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<td>Ubiquitination</td>
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<tr>
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<tr>
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<td>K225</td>
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</tr>
<tr>
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</tr>
<tr>
<td>127</td>
<td>Phosphorylation</td>
<td>Y291</td>
<td>STSPESPytHWkQtV</td>
</tr>
<tr>
<td>5</td>
<td>Phosphorylation</td>
<td>T292</td>
<td>TSPESPytHWkQtVF</td>
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<tr>
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<td>K324</td>
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</table>

*aAdapted from (Hornbeck et al. 2012)*
Figure 3.1 Structural comparisons between the PRMTs and contrast between amino acid side chains. (A) The hydroxyl of S217 of CARM1 hydrogen bonds to the backbone carbonyl of Y154 and is critical for activity. (B) The hydroxyl of S102 of PRMT1 (white) potentially hydrogen bonds to the backbone carbonyl of Y39. Note that because Y39 is not visible in the crystal structure of PRMT1, an overlay of PRMT3 (tan) was used to demonstrate its approximate location. (C) S102 was mutated to a glutamate and alanine residue to probe its function. This figure was prepared with UCSF Chimera using the coordinates from CARM1 (PDBID 3B3F), PRMT1 (PDBID 1ORI), and PRMT3 (PDBID 1F3L).

To address this issue in PRMT1, we set out to investigate the effect of phosphorylating S102 and Y291 on PRMT1 activity. As described above, S217 of CARM1 (S102 in PRMT1 numbering) is conserved amongst the Type I PRMTs and phosphorylation of this residue abolishes the methyltransferase activity of CARM1. Examination of the crystal structures of CARM1 and PRMT1 (with PRMT3 overlay because the N-terminal tail residues are absent in the PRMT1 structure) revealed the possibility that phosphorylation of this residue may be a common regulatory mechanism between both enzymes due to their similar positions (Figure 3.1A-B). In order to determine if phosphorylation of S102 in PRMT1 would yield the same effect as it does in CARM1, a S102E mutant was created, to mimic phosphorylation of the residue, and a S102A mutant, to probe the general importance of the hydroxyl group (Figure 3.1C).
Figure 3.2 Structure of PRMT1 and structural contrast of amino acid side chains. (A) Results of previous studies involving mutation of H293 suggest that the addition of a phosphate to Y291 would likely negatively affect methyltransferase activity. (B) Structural contrast of tyrosine and phosphorylated tyrosine clearly shows that the addition of a phosphate group adds both bulk and charge to the residue. Mutation to a phenylalanine can reveal the importance of the hydroxyl group, whereas, a glutamate mutant is often used to determine the effect of the addition of a negative charge. However, due to the large difference in overall size between phosphotyrosine and glutamate, the incorporation of p-carboxymethyl L-phenylalanine is a better overall mimic. This figure was prepared with UCSF Chimera using the coordinates from PRMT1 (PDBID 1ORI).

Y291 was specifically chosen for this investigation because it has the largest number of curated mass spectrometry hits according to PhosphoSitePlus® (Hornbeck et al. 2012). Based on the position of this residue in the structure of PRMT1, we hypothesized that phosphorylation would impact methyltransferase activity and/or protein-protein interactions (Figure 3.2A). This prediction was based on previous studies that showed that H293 is important for catalysis, possibly due to the formation of a salt bridge with D51 (Rust et al. 2011). Although hydrogen bond formation between Y291 and E47 can be ruled out due to the 5.0 Å distance between the two, the addition of a
negatively charged phosphate group would likely cause repulsion and consequently open up the SAM binding pocket, as well as destabilize residues that are important for catalysis, similar to the case with H293. Although glutamate mutations are often used as a mimic of phosphotyrosine, comparison of the two structures clearly demonstrates that there is a difference in both size and electronics. In order to overcome this problem, p-carboxymethyl-L-phenylalanine (pCMF) (Figure 3.2), a previously reported stable mimic of phosphotyrosine (Xie et al. 2007), was installed at this position using an established method of unnatural amino acid (UAA) incorporation that utilizes an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair developed by the Schultz lab (Young et al. 2010 and Xie et al. 2007). In addition to the above mutagenesis studies, PRMT1 was profiled against thirty-three kinase targets in order to determine possible kinases responsible for phosphorylation. These experiments were done in collaboration with Kinexus Bioinformatics Corporation.

Herein, we describe our efforts to decipher the means of regulation of PRMT1 activity. Site-directed mutagenesis and UAA incorporation was used to investigate the consequence of phosphorylation on S102 and Y291. In addition, PRMT1 was profiled against a variety of kinases to determine if the protein could be phosphorylated.

3.2 Materials and Methods

3.2.1 Chemicals

Iscove’s Modified Dulbecco’s Medium (IMDM) was purchased from (ATCC). Acrylamide, ammonium persulfate, beta-mercaptoethanol, sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Bio-Rad (Hercules, CA). Acetonitrile, (ethylenedinitrilo)tetraacetic
acid (EDTA), dimethylformamide (DMF), Fmoc-protected amino acids, methanol, phenylmethylsulfonyl fluoride (PMSF), trifluoroacetic acid (TFA), and Wang resin were purchased from EMD (Gibbstown, NJ). Dichloromethane, diethyl ether, sodium chloride, triisopropylsilane were purchased from Fisher Scientific (Pittsburgh, PA). Mutagenic primers were purchased from IDT Inc. (Coralville, IA). Fetal bovine serum (FBS) was purchased from Mediatech Inc. (Manassas, VA). Dithiothreitol (DTT), 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), and tricine were purchased from RPI (Mt. Prospect, IL). 4-(bromomethyl)phenylacetic acid, trimethylsilyl-diazomethane (TMSCH$_2$N$_2$), (diphenylmethylene) glycine ethyl ester, benzyl trimethylammonium hydroxide, dimethyl sulfoxide (DMSO), and piperidine were purchased from Sigma-Aldrich (St. Louis, MO). $^{14}$C-labeled SAM and $\gamma^{33}$P-labeled ATP were purchased from Perkin-Elmer and $^{14}$C-labeled BSA from Sigma-Aldrich. Triton X-100 and sodium acetate were purchased from VWR (West Chester, PA). Site-directed mutagenesis, the purification of PRMT1, peptide synthesis, and the gel-based methylation assay were performed according to the procedures outlined in Chapter 2.

### 3.2.1 Cell Culture Maintenance & Whole Cell Extract Preparation

K562 cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Mediatech Inc) at 37 °C and 5% CO$_2$. For immunoprecipitation experiments, cells were grown to confluency and either 10 µM MG132, 10 µM MG132 (Cayman Chemical-10012628) plus 1 mM orthovanadate (NEB-P0758S), or DMSO as a control was added to three separate flasks and incubated for 2 h at 37 °C and 5% CO$_2$. Cells were removed
and centrifuged for 5 min at 225 x g and 4 °C. After three washes with PBS, cells were lysed by a 30 min incubation at 4 °C in modified RIPA buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1 mM EDTA, 1% NP-40) in the presence of complete protease inhibitor cocktail (Roche-4693132), phosphatase inhibitor cocktail II (Sigma-P8465), and 1 mM orthovanadate. Lysed cells were centrifuged for 5 min at 3824 x g at 4 °C and the supernatant was removed as the whole cell extract. Protein concentration was determined by a DC™ Protein Assay (Bio-Rad).

3.2.2 Immunoprecipitation

K562 cell extracts were diluted to 4 µg/µL (1 mg total protein) in modified RIPA buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1 mM EDTA, 1% NP-40) in the presence of cOmplete protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail II (Sigma), and 1 mM orthovanadate (NEB). Extracts and antibody control (dilution buffer only) were incubated with 5 µL of 1 µg/µL anti-PRMT1 antibody (Bethyl – A300-722A) for 2 h with rotation at 4 °C. A 50 µL slurry of Protein G Sepharose 4 Fast Flow Beads (GE) in cell extract dilution buffer was added to each sample and incubated ON with rotation at 4 °C. Beads were washed three times with 200 µL of modified RIPA buffer. Proteins were eluted with 60 µL of SDS-loading dye and heated for 10 min at 95 °C. Samples and controls were separated by 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with anti-PRMT1 (Abcam – ab7027) or 4G10® Platinum anti-phosphotyrosine antibodies followed by incubation with goat anti-mouse HRP conjugate (Millipore – 12-349). Proteins were visualized using Pierce ECL western blotting substrate (Thermo Scientific).
3.2.5 Synthesis of p-carboxymethyl-L-phenylalanine (pCMF)

pCMF was synthesized by modification of an already established protocol (Xie et al. 2007). A solution of 4 g (17 mmol) of 4-(bromomethyl)phenylacetic acid in 78.4 mL of THF and 39.2 mL of methanol was cooled to 0 °C followed by the slow addition of 17 mL (34 mmol) of trimethylsilyl-diazomethane (TMSCH$_2$N$_2$). The reaction was stirred for 25 min at 0 °C, then the solvent was evaporated in vacuo and the mass of the product was verified by LCMS: expected = $m/z$ 242.99 (M + H$^+$), observed = $m/z$ 243.0 (M + H$^+$). A solution of the product (17 mmol), 266 mg (1.6 mmol) of potassium iodide (KI), and 4.54 g (17 mmol) of (diphenylmethylene) glycine ethyl ester in 77.5 mL of dioxane was cooled to 0 °C followed by the slow addition of 6.5 mL of benzyl trimethylammonium hydroxide (40% in water). The reaction was stirred at rt for 1 h and then extracted with dichloromethane (CH$_2$Cl$_2$) and washed with brine. Solvent was evaporated in vacuo and the mass of the product was verified by LCMS: expected = $m/z$ 430.19 (M + H$^+$), observed = $m/z$ 430.2 (M + H$^+$). Aqueous HCl (1N, 40 mL) was added to the product and stirred for 1 h at rt. The pH was then adjusted 8 with solid NaHCO$_3$ and the product was extracted with dichloromethane (CH$_2$Cl$_2$) and washed with brine. Solvent was evaporated in vacuo and the mass of the product was verified by LCMS: expected = $m/z$ 266.13 (M + H$^+$), observed = $m/z$ 266.1 (M + H$^+$). The compound was then dissolved in 30 mL of NaOH and 15 mL of THF and stirred overnight at rt. Solvent was evaporated in vacuo and product was partitioned in ether and water. The aqueous portions were collected and lyophilized to yield a sodium salt of the final product and the mass was verified by LCMS: expected = $m/z$ 224.08 (M + H$^+$), observed = $m/z$ 224.1 (M + H$^+$). The compound was used in subsequent experiments without further purification.
3.2.6 Incorporation of pCMF into PRMT1

Incorporation of pCMF was performed using methods adapted from previously reported protocols (Xie et al. 2007 & Young et al. 2010). Y291 of PRMT1 was mutated to a TAG stop codon using the site directed mutagenesis methods described in Chapter 1. The pEVOL-pCMF plasmid, a generous gift from Peter Schultz, was co-transformed into E. coli BL21(DE3) cells with the Y291TAG mutant. One colony was used to inoculate 5 mL of 2YT media containing 50 μg/mL kanamycin and 25 μg/mL chloramphenicol. Two liters of 2YT media were inoculated with 20 mL of overnight culture containing 50 μg/mL kanamycin and 25 μg/mL chloramphenicol and grown at 37 °C and 250 rpm until OD<sub>600</sub> = 0.8. The cells were supplemented with 1 mM pCMF before being induced with 0.2% arabinose and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then incubated with shaking at 30 °C overnight and the mutant protein was purified according to the established protocol for WT PRMT1 (see Chapter 2).

3.2.7 In-Gel Digest

The Y291pCMF mutant (5 μg) was separated by 12% SDS-PAGE. Coomassie stained gel bands corresponding to labeled PRMT1 were excised and the resultant gel pieces were washed and dehydrated two times. Existing disulfide bonds were reduced with 10 mM DTT and the resulting thiols were alkylated with 54 mM N-ethylmaleimide. Proteins were digested with trypsin (Promega – V5113), at a 1:10 (w:w)(trypsin:protein) ratio, overnight at 37 °C. The enzymatic reaction was quenched with 1% TFA and the resulting peptides were desalted and concentrated using C18 ZipTips (Millipore). Dried peptides were reconstituted in 0.1 % formic acid. All samples were analyzed by liquid chromatography-tandem MS (LC-MS/MS) using an EASY-nLC II system coupled to a
LTQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA). Briefly, peptides were concentrated and desalted on an RP trapping column (0.31 x 20 mm, ReproSil-Pur C18-AQ, ThermoScientific) and eluted on-line with an analytical RP column (0.075 x 100 mm, ReproSil-Pur C18-AQ, ThermoScientific), operating at 300 nl/min and using a 40-min gradient from 0% to 40% solvent B [solvent A: 0.1% formic acid (v/v), solvent B: 0.1% formic acid (v/v), and 80% acetonitrile (v/v)]. The LTQ was programmed in the ‘selected MS/MS ion monitoring’ (SMIM) mode. The m/z 400-1600 survey scan was first performed in order to check for the presence of digested peptides as well as peptide separation along the gradient. This survey scan is followed by dependent MS/MS scans that fragment the six most intense ions to confirm the presence of the protein of interest. Next, MS/MS spectra were programmed, focusing on m/z 641.64, which corresponds to the triply-charged precursor ion of the modified RTGFSTSPESPY*THWK peptide, and on m/z 883.90, corresponding to the doubly-charged precursor ion of the modified TGFSTSPESPY*THWK peptide. Note that the quadruple charge stems from a possible charge on the fragmented C21 peptide and that the MS/MS scans were repeated twice.

3.2.8 Protein Kinase Assays

Protein kinase assays with PRMT1 as the substrate were performed by Kinexus Bioinformatics Corporation (Vancouver, B.C. Canada). Briefly, 5 µL of γ-33P-labeled ATP (50 M, 0.8 Ci final) was added to a mixture of 10 µL of kinase assay buffer, 5 µL of an active protein kinase (~10-50 nM final), and 5 µL of PRMT1 or control substrate (1-5 g) to start the reaction. Samples were incubated at ambient temperature for 20-40 minutes (depending on the target). Assays were quenched by spotting 10 µL of the
reaction mixture onto a Multiscreen phosphocellulose P81 plate, which was then washed 3 times for approximately 15 minutes each in a 1% phosphoric acid solution. The radioactivity on the P81 plate was counted in the presence of scintillation fluid in a Trilux scintillation counter. A control assay for each kinase target included all assay components except the appropriate substrate (replaced with equal volume of assay dilution buffer). Both the sample and control assays were done in duplicate and the corrected activity for each kinase target was determined by subtracting the blank control value.

3.3 Results and Discussion

3.3.1 Effect of Phosphorylation of S102

Disruption of the interaction between the hydroxyl group of S102, a conserved residue amongst the type I PRMTs, and the back backbone carbonyl of Y39, also a conserved residue, by phosphorylation has been demonstrated to negatively regulate CARM1 (Feng et al. 2009). To investigate whether phosphorylation of this residue may be a mechanism of regulation for PRMT1, we generated the S102A mutant to determine the importance of the hydroxyl group, and a S102E mutant to mimic phosphorylation. As seen in Tables 3.2 and 3.3, the results show that neither mutant affects the kinetic parameters of PRMT1 to the degree that was observed with CARM1, where mutations appear to abolish activity. However, we did observe a ~ 4.5- and ~ 5.5-fold increase in the $K_{m}$ values for AcH4-21 with the S102E and S102A mutant; there was essentially no effect on $k_{cat}$, and the kinetic parameters for SAM are identical to WT. These small but significant increases can likely be attributed to the destabilization of the N-terminal helix, which we speculate plays a role in substrate recognition and binding. Overall, these
results show that it is highly unlikely that PRMT1 activity is regulated by phosphorylation at S102, however, modification of this residue cannot be ruled out as more dramatic effects on protein-protein interactions and cellular localization are possible.

Table 3.2 Kinetic parameters of PRMT1 mutants for the AcH4-21 peptide

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>1.1 ± 0.5</td>
<td>-----</td>
<td>4.6 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>-----</td>
<td>4.1 x 10$^5$</td>
<td>-----</td>
</tr>
<tr>
<td>S102E$^a$</td>
<td>5 ± 1</td>
<td>4.5</td>
<td>5.7 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>0.8</td>
<td>1.1 x 10$^5$</td>
<td>3.7</td>
</tr>
<tr>
<td>S102A$^a$</td>
<td>6 ± 1</td>
<td>5.5</td>
<td>6.3 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>0.7</td>
<td>1.1 x 10$^5$</td>
<td>3.7</td>
</tr>
</tbody>
</table>

$^a$[SAM] = 15 µM.

Table 3.3 Kinetic parameters of PRMT1 mutants for SAM

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>6 ± 1</td>
<td>-----</td>
<td>5.8 x 10$^{-1}$ ± 3 x 10$^{-2}$</td>
<td>-----</td>
<td>1 x 10$^5$</td>
<td>-----</td>
</tr>
<tr>
<td>S102E$^a$</td>
<td>5.1 ± 0.7</td>
<td>0.9</td>
<td>5.1 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>1.1</td>
<td>9.9 x 10$^4$</td>
<td>1.0</td>
</tr>
<tr>
<td>S102A$^a$</td>
<td>5.6 ± 0.6</td>
<td>0.9</td>
<td>5.2 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>1.1</td>
<td>8.8 x 10$^4$</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$[AcH4-21] = 100 µM.

3.3.2 Probing for Phosphotyrosine in PRMT1

As mentioned above, PhosphoSitePlus® (www.phosphosite.org) lists tyrosine phosphorylation as an observed modification to a variety of PRMT1 residues; this list is compiled from curated mass spectrometry data (Hornbeck et al. 2012). In an attempt to confirm these mass spectrometry results and determine a phenotype for the modification, we immunoprecipitated PRMT1 from K562 cells, a cell line from which a subset of
PhosphoSitePlus® data is derived. To aid in the enrichment of the phosphorylated enzyme, the cells were incubated with either DMSO as a control, MG132, a proteasome inhibitor, or MG132 and orthovanadate, a phosphatase inhibitor. MG132 was added because phosphorylation can often be an upstream signal for degradation. Orthovanadate was added to prevent the removal of the phosphate group and possibly lead to an accumulation of the modified protein. The enzyme was immunoprecipitated with an anti-PRMT1 antibody and the blots were probed with either an anti-PRMT1 or an anti-phosphotyrosine antibody (Figure 3.3). Unfortunately, phosphorylated PRMT1 was not detected under any of the conditions tested. Immunoprecipitations were also performed with the anti-phosphotyrosine antibody, however, the results were the same.

![Table and Image]

Figure 3.3 Immunoprecipitation of PRMT1. K562 cells were treated prior to lysis with DMSO, MG132, or a combination of MG132 and orthovanadate, and immunoprecipitation on cell extracts was performed with anti-PRMT1. The resultant blots were probed with either an anti-PRMT1 or an anti-phosphotyrosine antibody.
3.3.3 Effect of Phosphorylation of Y291

Although tyrosine phosphorylation was not detected in cell extracts, we sought to determine the effect that phosphorylation of Y291 could have on PRMT1 activity. We generated the Y291F mutant to determine the importance of the hydroxyl group, and the Y291E mutant to mimic phosphorylation. However, because of the size and electronic difference between glutamate and phosphotyrosine (Figure 3.2), we decided to generate a better mimic. To do this, the Y291 position on PRMT1 was mutated to a TAG stop codon and pCMF, a known stable mimic of phosphorylated tyrosine (Xie et al. 2007), was synthesized according to the scheme outlined in Figure 3.4. pCMF was incorporated into the protein using an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair. Figure 3.51 shows a comparison between the expression of WT PRMT1 and the Y291pCMF mutant under the same conditions from a test expression in *E. coli* BL21(DE3) cells. The expression was then scaled up (Figure 3.6A) and the Y291pCMF mutant was purified according to the WT PRMT1 protocol (Figure 3.6B). Note that the Y291TAG truncation mutant did not co-purify with the Y291pCMF mutant, likely due to the inability to fold properly. The presence of pCMF at this position was confirmed by MS/MS (Figure 3.7).
Figure 3.5 Incorporation of pCMF into PRMT1. Test expressions were performed with the WT enzyme and the Y291TAG mutant in the presence of pCMF in *E. coli*.

Figure 3.6 Expression and purification of PRMT1(Y291pCMF). (A) Expression conditions were scaled up to 2 L and incorporation of pCMF was consistent with the test expressions. (B) The mutant was purified according to the WT PRMT1 protocol and yielded only the full length enzyme.

The kinetic parameters for the Y291 mutants can be found in Tables 3.4 and 3.5. In regards to AcH4-21, the Y291F and Y291E mutants yielded a ~ 2.0-fold and ~ 1.4-fold decrease in $k_{\text{cat}}/K_m$, respectively, compared to the WT enzyme, and these observed decreases were solely based on small increases in $K_m$. The Y291pCMF mutant, however, yielded a more significant decrease (~ 5.1-fold) in $k_{\text{cat}}/K_m$ that was caused by an ~ 7.3-fold increase in $K_m$. These results suggest that the Y291 plays a small role in substrate binding, but not catalysis, and thus phosphorylation of this residue would likely affect substrate binding.
Figure 3.7 MS/MS Analysis of PRMT1(Y291pCMF).

Table 3.4 Kinetic parameters of PRMT1 mutants for the AcH4-21 peptide

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>1.1 ± 0.5</td>
<td>-----</td>
<td>4.6 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>-----</td>
<td>4.1 x 10$^5$</td>
<td>-----</td>
</tr>
<tr>
<td>Y291F$^a$</td>
<td>5 ± 1.8</td>
<td>4.6</td>
<td>1.03 ± 6 x 10$^{-2}$</td>
<td>0.45</td>
<td>2.1 x 10$^5$</td>
<td>2.0</td>
</tr>
<tr>
<td>Y291E$^a$</td>
<td>4.4 ± 0.6</td>
<td>4.0</td>
<td>1.32 ± 3 x 10$^{-2}$</td>
<td>0.35</td>
<td>3.0 x 10$^5$</td>
<td>1.4</td>
</tr>
<tr>
<td>Y291pCMF$^a$</td>
<td>8 ± 1.8</td>
<td>7.3</td>
<td>6.4 x 10$^{-1}$ ± 2 x 10$^{-2}$</td>
<td>0.72</td>
<td>8.0 x 10$^4$</td>
<td>5.1</td>
</tr>
</tbody>
</table>

$^a$[SAM] = 15 µM.

Table 3.5 Kinetic parameters of PRMT1 mutants for SAM

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>6 ± 1.0</td>
<td>-----</td>
<td>5.8 x 10$^{-1}$ ± 3 x 10$^{-2}$</td>
<td>-----</td>
<td>1 x 10$^5$</td>
<td>-----</td>
</tr>
<tr>
<td>Y291F$^a$</td>
<td>8 ± 1.0</td>
<td>1.3</td>
<td>1.55 ± 6 x 10$^{-2}$</td>
<td>0.37</td>
<td>1.8 x 10$^5$</td>
<td>0.56</td>
</tr>
<tr>
<td>Y291E$^a$</td>
<td>7 ± 1.5</td>
<td>1.2</td>
<td>1.6 ± 1 x 10$^{-1}$</td>
<td>0.36</td>
<td>2.3 x 10$^5$</td>
<td>0.43</td>
</tr>
<tr>
<td>Y291pCMF$^a$</td>
<td>6 ± 1.1</td>
<td>1.0</td>
<td>5.5 x 10$^{-1}$ ± 3 x 10$^{-2}$</td>
<td>1.1</td>
<td>9.1 x 10$^4$</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$[AcH4-21] = 100 µM.
Surprisingly, the kinetic parameters for all three mutants matched those of the WT enzyme. This was puzzling because, based on the crystal structure of PRMT1 and previous studies with H293 (Figure 3.2A), we hypothesized that disruption of the N-terminal α-helix would greatly impact SAM binding. However, these results demonstrate that the Y291 residue does not play a role in SAM binding and therefore phosphorylation of this residue would likely not affect SAM binding or catalysis.

3.3.4 Profiling of PRMT1 Against Select Kinases

Because we were unable to detect phosphorylated PRMT1 in vivo, we decided to take a different approach by profiling PRMT1 against select kinases to determine if PRMT1 can be phosphorylated in vitro. Radioactive kinase assays were performed by Kinexus Bioinformatics Corporation with our PRMT1 enzyme. Thirty-three kinases were assayed in total and the twenty-five kinases with the most activity towards PRMT1 can be found in Table 3.6. There are two different methods for comparing activity. The first method is to examine the percent change from control (% CFC) with percentages > 5% being significant. This method suggests that PRMT1 is a likely substrate for cKIT, KDR, ERK1, ERK5, JNK1, JNK2, JNK3, FLT1, and AXL. This method can be misleading, however, because the control substrates are often optimized for the particular kinase and/or used in high concentrations. The second method only examines the CPM counts for PRMT1 that pertain to each kinase, with counts >8000 being significant. Note that the kinases displayed in Table 3.6 were chosen based on the second method. This method suggests that PRMT1 is a likely substrate for AXL, KDR, FGR, FYN A, SRC, YES1, FER, and LYN A, which are all tyrosine kinases. These results are promising in that they provide a starting point for future in vitro and in vivo kinase experiments.
Table 3.6 Percent activity of select protein kinases with PRMT1

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Substrate CPM</th>
<th>PRMT1 CPM</th>
<th>% CFC</th>
</tr>
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<tbody>
<tr>
<td>AXL</td>
<td>303327</td>
<td>27780</td>
<td>9</td>
</tr>
<tr>
<td>KDR</td>
<td>142513</td>
<td>14876</td>
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<tr>
<td>FGR</td>
<td>332996</td>
<td>13096</td>
<td>4</td>
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<tr>
<td>FYN A</td>
<td>341735</td>
<td>13058</td>
<td>4</td>
</tr>
<tr>
<td>SRC</td>
<td>305044</td>
<td>11629</td>
<td>4</td>
</tr>
<tr>
<td>YES1</td>
<td>389767</td>
<td>11474</td>
<td>3</td>
</tr>
<tr>
<td>FER</td>
<td>454478</td>
<td>8759</td>
<td>2</td>
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<tr>
<td>LYN A</td>
<td>347599</td>
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<tr>
<td>FES</td>
<td>210964</td>
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<td>3</td>
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<tr>
<td>HCK</td>
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<tr>
<td>FLT1</td>
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<td>6032</td>
<td>5</td>
</tr>
<tr>
<td>LCK</td>
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<td>4</td>
</tr>
<tr>
<td>FLT3</td>
<td>134185</td>
<td>5310</td>
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<td>FGFR3</td>
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<td>ERK5</td>
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<td>FRFR1 (FLT2)</td>
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<td>JNK2</td>
<td>27479</td>
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<tr>
<td>FGFR4</td>
<td>136640</td>
<td>2495</td>
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<td>25072</td>
<td>1653</td>
<td>7</td>
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<tr>
<td>p38a MAPK</td>
<td>248961</td>
<td>1011</td>
<td>0</td>
</tr>
</tbody>
</table>
3.4 Conclusions

Post-translational modification of PRMT1 has yet to be characterized; therefore we set out to investigate the regulation of the enzyme by PTMs, specifically phosphorylation. Our research efforts focused on two residues, S102 and Y291. S102 is conserved in Type I PRMTs and phosphorylation of this residue is a means of regulation for CARM1. Two mutants, S102A and S102E, were made in PRMT1 and the kinetic analysis of these mutants demonstrated that this residue is not important for substrate or cofactor binding, or catalysis, and thus it is highly unlikely that PRMT1 is regulated by a mechanism similar to CARM1.

The Y291 residue was chosen for investigation based on curated mass spectrometry data from PhosphoSitePlus® (Hornbeck et al. 2012). Y291F and Y291E mutants were generated, as well as the incorporation of pCMF into the protein, in an attempt to properly mimic phosphotyrosine. The results showed that, in general, this residue likely plays a minor role in substrate binding and that phosphorylation at this position would likely disrupt substrate binding. However, to our surprise, no affect was observed in regards to SAM, which was quite puzzling based on structural observations and previous studies with H293. These results suggest that the interaction between H293 and D51 is likely enough to hold the main portion of the α-helix in place, despite the addition of a phosphate group at Y291. Interestingly, based on structure analysis, the interaction between histidine and aspartate appears to be conserved amongst Type I PRMTs, and thus it is likely that this is a critical interaction for PRMT catalysis. In addition, as mentioned in Chapter 1, PRMT1 has a variety of splice variants and these variations at the N-terminus appear to play a role in substrate specificity (Goulet et al.
Therefore, it is possible that phosphorylation of Y291 could modulate substrate specificity by affecting the position of the N-terminal tail. Note that the pCMF residue is still lacking a negative charge compared to phosphotyrosine so it is possible that the modification may have an even greater effect in vivo.

In collaboration with Kinexus Bioinformatics Company, we have profiled PRMT1 against thirty-three different protein kinases to determine if the enzyme can be phosphorylated in vitro. The results show that several tyrosine kinases (i.e., AXL, KDR, FGR, FYN A, SRC, YES1, FER, and LYN A) are active against PRMT1. Future experiments will include in vitro assays with the top purified kinases to attempt to determine potential sites of modification. In vivo experiments will also be performed in which stimuli will be added in order to activate signaling pathways that are particular for the most active kinases, thus possibly increasing the concentration of phosphorylated PRMT1.
CHAPTER 4

Regulation of PRMT1 by Protein-Protein Interactions

4.1 Introduction

As discussed in Chapter 3, it is highly likely that the PRMTs are regulated by a variety of mechanisms (i.e., the activation or repression of gene expression, PTMs, and/or protein-protein interactions). The role of PTMs in regards to PRMT1 activity was discussed in Chapter 3, but we also were interested in determining how protein-protein interactions regulate the activity of this enzyme because preliminary data indicates that the PRMTs are regulated via this mechanism. For example, there is evidence that the tumor suppressor protein DAL-1/4.1B enhances PRMT3-dependent methylation of MCF-7 cell lysates (Singh et al. 2004), however, it both enhances and inhibits PRMT5 dependent methylation in a substrate specific manner (Jiang et al. 2005). In another example, the substrate specificity of CARM1 is altered by BRG1, a component of the nucleosomal methylation activator complex (NUMAC), which shifts CARM1-dependent methylation towards nucleosomal histone H3 as opposed to the free histone (Xu et al. 2004). In addition, PRMT5 is regulated by its binding partner MEP50, as there is a substantial difference in methyltransferase activity between the enzyme alone and the enzyme in a complex with MEP50. PRMT5 is also active as part of a variety of multi-protein complexes, that include the 20S methylosome and the Swi/Snf complex, with MEP50 being a common component (Antonysamy et al. 2012).
Although still not fully understood, there is evidence that PRMT1 activity is regulated by protein-protein interactions. The isozyme was originally identified due to its interaction with the anti-proliferative proteins BTG1 and BTG2 (Lin et al. 1996) and subsequent experiments suggested that these two proteins are capable of enhancing PRMT1 activity (Lin et al. 1996 & Berthet et al. 2002). More recently, the deadenylase CCR4 Associated Factor 1 (CAF1) was found to modulate enzyme activity in a substrate specific manner by decreasing methylation of SAM68 and histone H4 but not affecting hnRNPA1 methylation \textit{in vitro}. These observations were validated \textit{in vivo} using siRNA knockouts of CAF1 in MCF-7 cells and probing for asymmetric dimethylation of SAM68 and histone H4. The authors also showed that \textit{in vitro}, BTG1 increases PRMT1-dependent methylation of Histone H4 while decreasing methylation of SAM68. In addition, the authors were able to pull down CAF1 and BTG1 with GST-PRMT1 and show that PRMT1 and CAF1 colocalize in the cell (Robin-Lespinasse et al. 2007). Interestingly, BTG1 and CAF1 are binding partners (Rouault et al. 1998 and Bogdan et al. 1998), thus providing further evidence for a likely regulatory mechanism involving all three proteins.

Because the initial studies examining the effect of BTG1 and CAF1 on PRMT1 activity only looked at relative rates, we set out to perform a more in depth characterization and thereby provide a molecular basis for the regulation afforded by these interacting proteins. Using the PRMT1 catalyzed asymmetrical dimethylation of histone H4 at R3 as a model (Figure 4.1A), at least two possible scenarios exist for the regulation of PRMT1 by its interacting partners (Figure 4.1B). In both cases, the interacting protein (i.e., CAF1 or BTG1) could bind to PRMT1 or the substrate. In the
case of CAF1, binding would prohibit methylation, however, in the case of BTG1, binding would recruit PRMT1 to the target arginine, in this case on histone H4. The role that both CAF1 and BTG1 play together in the regulation of PRMT1 has yet to be determined. To probe the illustrated scenarios, PRMT1 activity assays were performed at increasing concentrations of CAF1 and BTG1, individually and together, with low and high concentrations of histone H4 and AcH4-21, a minimal peptide substrate of histone H4. The results suggest that CAF1 can bind histone H4 and prevent methylation and/or it can bind PRMT1 and target it to histone H4. In addition, it is likely that BTG1 binds to both proteins as well; however, it appears to have less affinity for histone H4 compared to CAF1.

Figure 4.1 Enzyme regulation scenarios of PRMT1 by CAF1 and BTG1. (A) PRMT1 asymmetrically dimethylates histone H4 at R3. (B) CAF1 and BTG1 can exhibit their regulatory effects on PRMT1 by either binding the enzyme or binding the potential substrate.
4.2 Materials and Methods

4.2.1 Chemicals

Acrylamide, ammonium persulfate, beta-mercaptoethanol, sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Bio-Rad (Hercules, CA). Acetonitrile, (ethylenedinitrilo)tetraacetic acid (EDTA), dimethylformamide (DMF), Fmoc-amino acids, methanol, phenylmethylsulfonyl fluoride (PMSF), trifluoroacetic acid (TFA), and Wang resin were purchased from EMD (Gibbstown, NJ). Dichloromethane, diethyl ether, glutathione (reduced), imidazole, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, triisopropylsilane (TIS), and urea were purchased from Fisher Scientific (Pittsburgh, PA). Dithiothreitol (DTT), 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), and tricine were purchased from RPI (Mt. Prospect, IL). Guanidinium hydrochloride, and piperidine were purchased from Sigma-Aldrich (St. Louis, MO). \(^{14}\)C-labeled SAM was purchased from Perkin-Elmer and \(^{14}\)C-labeled BSA from Sigma-Aldrich. Triton X-100 and sodium acetate were purchased from VWR (West Chester, PA). The purification of PRMT1 and the synthesis of the AcH4-21 peptide are outlined in Chapter 2.

4.2.2 Purification of Histone H4

Recombinant histone H4 was purified with slight modification to an established protocol (Thompson et al. 2001). A plasmid encoding histone H4 was transformed into E. coli BL21(DE3) cells and starter cultures, prepared from single colonies in the presence of 100 µg/mL ampicillin, were incubated overnight at 37 °C with rotation. Overnight starter cultures were then used to inoculate 2 L of 2YT media plus ampicillin.
(32 g tryptone, 20 g yeast, 10 g NaCl, 100 µg/mL ampicillin) and the culture was grown at 37 °C with shaking to an OD$_{600}$ of 0.4. Protein expression was induced with 0.4 mM IPTG for 1.5 h. Cells were harvested by centrifugation at 4400 x g for 10 min and the pellet was resuspended in Wash Buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF) followed by flash freezing in liquid nitrogen. The cell suspension was thawed at 42 °C for 30 min and cells were lysed by 8 cycles of sonification (8 sec pulse with 1 min rest at 100% amplitude). Lysed cells were centrifuged for 20 min at 23,000 RPM and the resulting pellet was washed with 50 mL of Wash Buffer plus 1% Triton X-100. The washed pellet was centrifuged for 10 min at 14,000 x g, the supernatant was discarded, and a second round of washing and centrifugation was performed. The final pellet was minced in 1 mL of DMSO and incubated at RT for 30 min followed by the addition of 10 mL of unfolding buffer (7 M guanidinium HCl, 20 mM Tris pH 7.5, 10 mM DTT). The resuspension was incubated for 1 h at RT with stirring then centrifuged for 10 min at 13,000 x g and the supernatant was removed and saved. The pellet was resuspended in 5 mL of unfolding buffer, incubated for another 10 min at RT with stirring, and centrifuged for 10 min at 13,000 x g. The second supernatant was combined with the first and dialyzed overnight against 4 L of ddH$_2$O and 1 mM β-mercaptoethanol at 4 °C. The solution was lyophilized the following day and the dried white powder was resuspended in 20 mM sodium acetate pH 5.2, 5 mM β-mercaptoethanol, 1 mM EDTA, 7 M deionized urea and further purified using a CM Sepharose™ Fast Flow (GE Healthcare) column on the FPLC, with a stationary phase of 20 mM sodium acetate pH 5.2, 5 mM β-mercaptoethanol, 1 mM EDTA, and 5 mM deionized urea and a linear gradient mobile phase of 20 mM sodium acetate pH 5.2, 5
mM β-mercaptoethanol, 1 mM EDTA, 5 mM deionized urea, and 2 M sodium chloride. Fractions were checked by SDS-PAGE and pure fractions were dialyzed against 4 L of ddH2O and 1 mM β-mercaptoethanol at 4 °C and subsequently lyophilized the next day. The dried protein was dissolved in unfolding buffer to a concentration of 2 mg/mL and incubated for 15 min at RT followed by a two-fold dilution in unfolding buffer and incubation for 1 h at RT. Histone H4 was then dialyzed overnight against 10 mM Tris pH 7.5, 250 mM sodium chloride, 1 mM EDTA, and 5 mM β-mercaptoethanol at 4 °C.

4.2.3 Purification of mCAF1, hBTG1, and Glutathione S-transferase

The mCAF1 and hBTG1 plasmids were a generous gift from Dr. Muriel Le Romancer and were subcloned from their original pGEX-ET E. coli expression vectors into pGEX-6P-1 (BamHI and SmaI restriction sites) and pGEX-6P-2 (EcoRI restriction sites) E. coli expression vectors, respectively. The plasmids were separately transformed into E. coli Rosetta cells and single colonies were used to inoculate starter cultures, in the presence of 80 µg/mL ampicillin and 25 µg/mL chloramphenicol, that were grown overnight at 37 °C. Starter cultures, in addition to 50 µg/mL ampicillin and 17.5 µg/mL chloramphenicol, were added to 2 x 1 L of TB media (12 g tryptone, 24 g yeast, 0.4% glycerol, 0.17 M KH2PO4, and 0.72 M K2HPO4) and grown at 37 °C with shaking until an OD600 of 0.8-1.0 was achieved. Protein expression was induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside and cultures were incubated at 16 °C overnight. Cells were harvested by centrifugation at 4,400 x g for 10 min at 4 °C. The pellet was resuspended in 26 mL of lysis buffer (20 mM Tris pH 8, 5 mM EDTA, 20% glycerol, 400 mM sodium chloride, and 5 mM DTT) and 4 mL of protease inhibitor cocktail solution (72 mg of Protease Inhibitor Cocktail (Sigma – P8465), 800 µL of dimethyl sulfoxide,
3200 µL of ddH\(_2\)O). The resuspended pellet was mixed slowly for 30 min at 4 °C followed by 12 cycles of sonication (15 sec pulse with 1 min rest at 100% amplitude). Lysed cells were centrifuged at 23,000 x g for 30 min at 4 °C. The supernatant was applied to a Glutathione Sepharose® 4B (GE Healthcare) column followed by a low salt wash (20 mM Tris pH 8, 1 mM EDTA, 20% glycerol, 200 mM sodium chloride, and 1 mM DTT) and a high salt wash (20 mM Tris pH 8, 1 mM EDTA, 20% glycerol, 500 mM sodium chloride, and 1 mM DTT). The proteins were then eluted with glutathione buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 8 and 1 mM DTT). Washes and elution fractions were checked by SDS-PAGE and PreScission Protease (GE Healthcare) was added to the fractions containing protein prior to overnight dialysis against 20 mM Tris pH 8, 20 mM EDTA, 1 mM DTT, and 500 mM sodium chloride 4 °C. Dialyzed protein was applied to a regenerated Glutathione Sepharose® 4B column and the flow through was collected and dialyzed for 3 h against 20 mM Tris pH 8, 1 mM EDTA, and 1 mM DTT 4 °C. The protein was further purified using a HiTrap™ Q Fast Flow (GE Healthcare) column on the FPLC, with a stationary phase of 20 mM Tris pH 8.5 and 2 mM DTT and a linear gradient mobile phase of 10 mM Tris pH 8.5, 2 mM DTT, and 1 mM sodium chloride. Fractions were checked by SDS-PAGE and pure fractions were dialyzed against 20 mM Hepes pH 8, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 5 mM sodium chloride at 4 °C.

Glutathione S-transferase (GST) was purified using a modified mCAF1 and hBTG1 protocol. The empty pGEX-6P-1 vector was used to express GST and the pure protein was collected in the glutathione buffer fraction (10 mM reduced glutathione, 50 mM Tris-HCl pH 8 and 1 mM DTT) from the Glutathione Sepharose® 4B (GE Healthcare) column.
Healthcare) column. The protein was then dialyzed against 20 mM HEPES pH 8, 1 mM EDTA, 1 mM DTT, 10% glycercol, and 5 mM sodium chloride at 4 °C.

4.2.4 Gel-Based Methylation Assay

The gel-based PRMT1 methylation assay has been previously described (Osborne et al. 2007). For percent activity experiments in which the enzyme was preincubated with the interacting proteins, reaction mixtures of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM sodium chloride, 0.5 mM dithiothreitol, 15 μM [14C]-labeled SAM, 0.2 μM PRMT1 or 0.8 μM PRMT1, and varying concentrations of GST, mCAF1, hBTG1, or mCAF1/hBTG1 (0-30 μM) were preincubated at 37 °C for 10 min. The reaction was initiated with 25 μM AcH4-21, 3 μM histone H4, or 25 μM histone H4 and was quenched after 15 min or 90 min. For percent activity experiments in which the substrate was preincubated with the interacting proteins, the same reaction mixture was preincubated with either 15 μM AcH4-21 or 3 μM histone H4 at 37 °C for 10 min. The reaction was initiated with 0.8 μM PRMT1 and was quenched after 90 min. The samples were run on tris-tricine gels and incorporated radioactivity was quantified using Image Quant (Molecular Dynamics). The assays were done in duplicate and the standard deviation of the raw data values agreed within ≤ 20%. Percent activity was determined by dividing the rates at each particular interacting protein concentration by the acquired rate at 0 μM interacting protein.

4.3 Results and Discussion

Previous studies have qualitatively determined that PRMT1 activity is affected by CAF1 and BTG in a substrate specific manner (Lin et al. 1996, Berthet et al. 2002, Robin-Lespinasse et al. 2007). In order to quantitatively analyze these effects, as well as
Figure 4.1 Effect of interacting proteins on PRMT1 activity at high concentrations of substrate. Enzyme was preincubated with the interacting protein(s) followed by initiation of the methylation reaction with (A) histone H4 (25 µM) or (B) AcH4-21 (25 µM).

To determine the possible mechanism of regulation (Figure 4.1), we performed in vitro activity assays with PRMT1 in the presence of CAF1, BTG1, and a combination of the two. These assays were done at low and high concentrations of both histone H4 and the AcH4-21 peptide. Because the original studies demonstrated that CAF1 and BTG1 co-immunoprecipitate with PRMT1, the first assays that were performed involved preincubating PRMT1 with the interacting protein(s) followed by initiation of the methylation reaction with a high concentration of substrate (Figure 4.2). The results show a general increase in activity in the presence of the interacting proteins, individually and together, as well as for the GST control, for both histone H4 and the AcH4-21 peptide. These results were confusing as they do not agree with previously published results, which show that CAF1 inhibits and BTG1 enhances methylation of histone H4. Therefore, the assay was repeated in the same manner but with a lower concentration of
histone H4, similar to that of the original study, to determine if substrate concentration was a factor (Figure 4.3). Here, an increase in activity in the presence of CAF1, BTG1, and a combination of both was observed, followed by a decrease starting at 10 µM, while the GST control remained constant. Although inhibition was observed, these results were still not consistent with previous findings and thus unsatisfying.

**Figure 4.3** Effect of interacting proteins on PRMT1 activity at low concentrations of substrate. Enzyme was preincubated with the interacting protein(s) followed by initiation of the methylation reaction with histone H4 (3 µM).

To determine if the two proteins affect PRMT activity by binding to the substrate instead (Figure 4.1), assays were repeated except with preincubation of the interacting proteins and the substrates followed by initiation of the reaction with PRMT1 (Figure 4.4). The results observed with histone H4 were more like the results that we expected, with a decrease in activity in the presence of CAF1 and a slight increase with BTG1.
Quite surprisingly, however, there was generally no inhibition or activation with AcH4-21 as the substrate. This observation suggests that the interacting proteins likely bind to the core of histone H4, which is absent from the peptide. In addition, the presence of both CAF1 and BTG1 decreased PRMT1 activity, almost to the extent of CAF1. Conclusions cannot yet be made from this observation however, as more experiments will need to be done to probe this interaction.

Figure 4.4 Effect of interacting proteins on PRMT1 activity at low concentrations of substrate. (A) Histone H4 (3 µM) or (B) AcH4-21 (15 µM) was preincubated with the interacting protein(s) followed by initiation of the methylation reaction with PRMT1.

A summary of the individual results with CAF1 and BTG1 can be found in Figure 4.5. It is obvious from these graphs that the preincubation conditions play a role in the effect of CAF1 on PRMT1 activity, \textit{i.e.}, inhibition when preincubated with histone H4 and activation when preincubated with PRMT1. This further demonstrates the likelihood that CAF1 can bind PRMT1, but may also be a histone binding protein, which has not been previously observed. The preincubation conditions only yield a very small
difference in regards to BTG1, which suggests that if BTG1 binds to histone H4 it is likely not a strong interaction.

Figure 4.5 Summary of the effects of CAF1 and BTG1 on PRMT1 activity. The red bars show results for the preincubation with histone H4 (3 µM) and the blue bars show for preincubation with PRMT1 with increasing concentrations of (A) CAF1 and (B) BTG1.

4.4 Conclusions

Studies have shown that CAF1 and BTG1 are capable of inhibiting or enhancing PRMT1 activity in a substrate dependent manner, although the exact mechanism of this regulation is unknown (vide supra). Therefore, efforts were made to quantify these effects and possibly determine the mechanism of inhibition or activation of PRMT1. Activity assays were performed with low and high concentrations of histone H4 and AcH4-21 and with varying concentrations of interacting proteins. The results show that preincubation of PRMT1 with the interacting protein(s) followed by initiation with a high concentration of substrate only enhances activity. The same assay repeated at lower concentrations of substrate showed some inhibition but not to the degree described by other groups. However, when histone H4 was preincubated with the interacting proteins, the results were similar to the previously reported results with inhibition caused by CAF1.
and activation by BTG1. Surprisingly, there was no effect on activity when AcH4-21 was preincubated with the proteins, which suggests that the proteins likely bind to the core of histone H4.

Looking at the possible scenarios for protein-protein interactions (Figure 4.1), the results allude to a possible scenario where CAF1 can bind PRMT1 and target it to histone H4, but can also bind histone H4 and prevent methylation. In the case of BTG1, it appears as though it can bind both proteins but has a lower affinity for histone H4 compared to CAF1. The lack of an effect with AcH4-21 further demonstrates the likelihood that both of these proteins bind histone H4.

Overall, initial efforts have been made to characterize the regulation of PRMT1 by its interacting partners. There is still a lot of work that needs to be done to determine their exact role in vivo. The fact that the effects on PRMT1 appear to be substrate dependent (Robin-Lespinasse et al. 2007) suggests that interaction with the substrates is critical for these observations. Therefore, studies with other substrates (e.g. SAM68) and co-immunoprecipitation experiments may shed light on whether the observed effects are mainly due to interactions with the substrate or with PRMT1. It would also be interesting to examine if the PRMT1 splice variants yield different outcomes with these interacting proteins.
CHAPTER 5

Development of PRMT1 Inhibitors

5.1 Introduction

As described in detail in Chapter 1, dysregulation of PRMT1 likely plays a role in breast cancer (Le Romancer et al. 2008 & Baldwin et al. 2012), leukemia (Cheung et al. 2007 & Shia et al. 2012), heart disease (Vallance et al. 2004), and ALS (Tradewell et al. 2012), thus suggesting that it may be a viable drug target. In addition, the precise roles that PRMT1 plays in normal cells have yet to be fully characterized, further demonstrating the usefulness of a specific inhibitor. The structural similarities between the PRMT family members as well as between the PRMTs and protein lysine methyltransferases (PKMTs), however, presents a difficult challenge when attempting to develop a potent and selective inhibitor or inactivator for PRMT1, as there are currently no cell permeable selective inhibitors to date. However, this shortcoming should not overshadow the efforts that have been made by various groups in the last decade to solve this predicament.

Arginine methyltransferase inhibitor 1, more commonly known as AMI-1, was the first PRMT selective inhibitor to be described. This compound, along with several less potent and/or selective compounds, was identified by screening a small molecule library (Figure 5.1). With an IC\textsubscript{50} value of 8.81 µM for PRMT1, the compound was
found to reversibly inhibit all of the PRMTs tested, but not the PKMTs, and shown to be cell permeable (Cheng et al. 2004). The lack of an effect on SAM binding in UV crosslinking experiments suggested that AMI-1 likely binds in the arginine binding pocket (Cheng et al. 2004) and this hypothesis was confirmed by inhibition studies using a fluorescence based assay (Feng et al. 2009). However, others have suggested that, according to molecular models, it binds partially in the SAM binding pocket and partially in the arginine binding pocket (Ragno et al. 2007). Centering on AMI-1 and several other compounds identified in the original library screen (i.e., AMI-5, AMI-6, and AMI-9) various groups have attempted to modify these structures in order to improve their potency and selectivity towards the PRMTs (Ragno et al. 2007, Mai et al. 2007, Bonham

Figure 5.1 Structures of selected PRMT inhibitors discovered from library screenings.
et al. 2010, Castellano et al. 2010). These analogues include substitutions of carboxylic groups for the sulfonic groups (e.g., 1b) (Castellano et al. 2010), substituted 1,5-diphenyl-1,4-pentadien-3-ones (e.g., 8) (Mai et al. 2007), as well as combining parts of AMI-1, AMI-6, and AMI-9 into one molecule (e.g., 4) (Bonham et al. 2010) (Figure 5.1). Although some of these compounds were found to be more potent than the original compounds, specificity was still lacking.

Although some of these compounds were found to be more potent than the original compounds, specificity was still lacking.

![Stilbamidine](image1.png)

![Allantodapsone](image2.png)

![RM-65](image3.png)

![C-7280948](image4.png)

![2e](image5.png)

![3](image6.png)

Figure 5.2 Structures of selected PRMT inhibitors discovered from virtual screenings.

A second and more targeted approach has also been undertaken by way of virtual screening using the structures of PRMT1, PRMT3, and CARM1 as guides (Spannhoff et al. 2007, Spannhoff et al. 2007, Heinke et al. 2009). These screens have produced compounds such as stillbamidine (Spannhoff et al. 2007), allantodapsone (Spannhoff et al. 2007), RM65 (Spannhoff et al. 2007), and C-7280948 (Heinke et al. 2009), as well as
subsequent analogs of these original compounds such as 2e (Bissinger et al. 2011) (Figure 5.2). In addition, a series of compounds, which possess the AMI-1 core, (e.g., 3) were found that target the substrates of PRMT1 as opposed to the enzyme itself (Feng et al. 2010) (Figure 5.2); this later finding raises questions regarding the inhibition mechanism of the aforementioned sulfonylureas. In spite of the fact that the aforementioned compounds are suitable inhibitors, and in some cases are cell permeable, their specificity amongst the members of the PRMT family has not been fully investigated.

Figure 5.3 Structures of SAM analogues and selected bisubstrate PRMT inhibitors.

Although, library and virtual screenings are popular techniques for the discovery of small molecule inhibitors, other groups have taken a more logical approach by modifying SAM and/or peptide versions of PRMT substrates for use as inhibitors. In support of this approach is the fact that S-adenosylhomocysteine (SAH), a product of
PRMT-dependent methylation, and sinefungin, a SAM analog, are potent inhibitors of the PRMTs, with specific IC₅₀’s for PRMT1 at 19.8 ± 5.57 µM and 1.44 µM ± 0.41 µM, respectively (Osborne et al. 2007) (Figure 5.3). Several bisubstrate analogs that incorporate both the SAM moiety and the target arginine residue have been characterized (e.g., 17 (Dowden et al. 2010) and 19 (Dowden et al. 2011)), with both showing specificity for the PRMTs over the PKTs and the latter showing specificity for PRMT1 over CARM1 (Figure 5.3). Another group has used a peptide based on the methylation site of fibrilarin as scaffolding for inhibitor design (e.g., R1-4) (Figure 5.3). In this case, substitutions were made at the Nη of the guanidinium group and the results show that these inhibitors are selective for PRMT1 and PRMT6 over CARM1 with IC₅₀ values less than 80 µM (Lakowski et al. 2010).

![Figure 5.4 Mechanism of PRMT1 inhibition by AAI.](image)

Our lab has also been focused on developing inhibitors for the PRMTs, specifically for PRMT1. The first inhibitor developed in our lab was 5’-(diaminobutyric acid)-N-iodoethyl-5’-deoxyadenosine ammonium hydrochloride (AAI) (Figure 5.4), with an IC₅₀ of 18.5 ± 4.2 µM in comparison to 350 ± 36 µM for AMI-1 (Osborne et al. 2008). Note that the large difference in the IC₅₀ values between our studies and the original AMI-1 publication are likely due to differences
in experimental conditions, (e.g., substrate identity and concentration), thus
demonstrating the error in comparing IC\textsubscript{50} values stemming from different conditions.
Although more laborious and time consuming, the best method for comparing
inhibition potency is $K_i$ or, in the case of irreversible inhibitors, $k_{inact}/K_i$.

![Figure 5.5 Structures of haloacetamidine based inhibitors and inactivators.](image)

**Table 5.1 IC\textsubscript{50} values for PRMT1 inhibitors and inactivators\textsuperscript{a}**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>C21 ($\mu$M)</th>
<th>Cl-amidine ($\mu$M)</th>
<th>F21 ($\mu$M)</th>
<th>F-amidine ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT1</td>
<td>1.8 ± 0.1</td>
<td>225 ± 10</td>
<td>94 ± 17</td>
<td>&gt;500</td>
</tr>
<tr>
<td>PRMT3</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>109 ± 28</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CARM1</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>PRMT6</td>
<td>8.8 ± 0.5</td>
<td>&gt;500</td>
<td>&gt;250</td>
<td>&gt;500</td>
</tr>
<tr>
<td>PAD4</td>
<td>145 ± 20</td>
<td>5.9 ± 0.3</td>
<td>117 ± 14</td>
<td>21.6 ± 2.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values from Obianyo 2010.

Target based inhibitor design in our lab has not been limited to the PRMTs.
In fact, our lab previously described two potent inactivators, Cl-amidine and F-amidine, for the PADs, a family of enzymes that convert arginine to citrulline in
proteins (Figure 5.5) (Luo et al. 2006a and Luo et al. 2006b). Because the PADs and the PRMTs both modify arginine residues, it was thought that these two compounds might also inhibit the PRMTs. The IC_{50} values for the compounds with PRMT1 were high (Table 5.1) (Obianyo et al. 2010), however, based on previous studies in our lab that demonstrated that positively charged residues distal to the site of modification are critical for substrate recognition (Osborne et al. 2007), it was likely that the lack of substrate recognition elements was the cause of this high value as opposed to the warhead. To test this hypothesis, the Cl- and F-acetamidine moieties were incorporated at the arginine 3 position of AcH4-21, a peptide substrate of PRMT1 that is based on the N-terminus of histone H4 (Figure 5.5) (Osborne et al. 2007). Indeed, the potency of the peptide based inhibitors, termed C21 and F21, was greatly increased and C21 was found to be selective for PRMT1 and PRMT6 over PRMT3 and CARM1 (Table 5.1). Interestingly, C21 was ~ 52-fold more potent than F21, and subsequent dialysis experiments demonstrated that C21 is an irreversible inactivator, however, F21 is a competitive reversible inhibitor. (Obianyo et al. 2010).

![Figure 5.6 Possible mechanisms of inactivation of the PRMTs.](image-url)
Possible mechanisms of inactivation of the PRMTs with the haloacetamidine-based inhibitors are illustrated in Figure 5.6. Further kinetic studies with C21 and PRMT1 revealed that inactivation for this isozyme is a two-step mechanism with $K_i$, $k_{\text{inact}}$, and $k_{\text{inact}}/K_i$ values of $\leq 0.8 \pm 0.4 \, \mu\text{M}$, $3.1 \pm 0.4 \, \text{min}^{-1}$, and $4.6 \times 10^6 \, \text{min}^{-1}\text{M}^{-1}$, respectively, thus making this the most potent PRMT1 inhibitor at this time (Obianyo et al. 2010). Studies with C21 also demonstrated inhibition of PRMT1 in cellulo (Obianyo et al. 2010), and the addition of a fluorescein or biotin tag, F-C21 and B-C21 respectively, transforms the inactivator into an activity-based probe that can selectively label or pull down PRMT1 from cell extracts (Obianyo et al. 2011).

Although C21 has been characterized extensively, the site of modification has remained a mystery. Substrate protection experiments, however, have suggested that it is likely an active site residue because the enzyme is protected from inactivation at higher substrate concentrations (Obianyo et al. 2010). Cl-amidine and F-amidine modify an active site cysteine residue in the PADs (Luo et al. 2006a and Luo et al. 2006b), however, the residue modified in PRMT1 is unknown; although a cysteine residue (C101) is present in the active site of PRMT1. Herein, we describe our efforts to determine the residue in PRMT1 that is modified by both Cl-amidine and C-21. We also describe our efforts to develop more potent and selective inhibitors for PRMT1 based on the site of modification. The results of these studies have opened new doors for the rational design of selective SAM-based inactivators of the PRMTs.

5.2 Materials and Methods

5.2.1 Chemicals

Acrylamide, ammonium persulfate, beta-mercaptoethanol, sodium dodecyl sulfate
(SDS), tetramethylethylenediamine (TEMED), and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Bio-Rad (Hercules, CA). Acetonitrile, (ethylenedinitrilo)tetraacetic acid (EDTA), dimethylformamide (DMF), Fmoc-protected amino acids, methanol, phenylmethylsulfonyl fluoride (PMSF), trifluoroacetic acid (TFA), and Wang resin were purchased from EMD (Gibbstown, NJ). 6-chloropurine riboside, dichloromethane, diethyl ether, N-ethylmaleimide, imidazole, sodium chloride, and triisopropylsilane (TIS) were purchased from Fisher Scientific (Pittsburgh, PA). Dithiothreitol (DTT), 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), and tricine were purchased from RPI (Mt. Prospect, IL). Piperidine was purchased from Sigma-Aldrich (St. Louis, MO). 14C-labeled SAM was purchased from Perkin-Elmer and 14C-labeled BSA from Sigma-Aldrich. The purification of PRMT1, site-directed mutagenesis, the synthesis of the AcH4-21 peptide, and the gel-based activity assay are outlined in Chapter 2. The synthesis of Cl-amidine (Luo et al. 2006a) and C-21 (Obianyo et al. 2010) have been previously described.

5.2.2 Labeling Reactions

For labeling of PRMT1 with Cl-amidine, a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, and 5 mM Cl-amidine was pre-incubated at 37 °C for 10 min. The reaction was initiated with 4.17 µM (5 µg) of PRMT1 and the reaction was quenched after 15 min with 4x SDS-loading dye. The entire reaction (5 µg of PRMT1) was separated by SDS-PAGE. For labeling of PRMT1 with C21, a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, and 8.33 µM C21 was pre-incubated at 37 °C for 10 min. The
reaction was initiated with 8.33 µM (10 µg) of PRMT1 and the reaction was quenched after 15 min with SDS-loading dye. Half of the reaction (5 µg of PRMT1) was separated by SDS-PAGE.

5.2.3 In-Gel Digestion

Coomassie stained gel bands corresponding to labeled PRMT1 were excised and the resultant gel pieces were washed and dehydrated two times. Existing disulfide bonds were reduced with 10 mM DTT and the resulting thiols were alkylated with 54 mM N-ethylmaleimide. Proteins were digested with trypsin (Promega – V5113), at a 1:10 (w:w)(trypsin:protein) ratio, overnight at 37 °C. The enzymatic reaction was quenched with 1% TFA and the resulting peptides were desalted and concentrated using C18 ZipTips (Millipore). Dried peptides were reconstituted in 0.1 % formic acid. All samples were analyzed by liquid chromatography-tandem MS (LC-MS/MS) using an EASY-nLC II system coupled to an LTQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA). Briefly, peptides were concentrated and desalted on an RP trapping column (0.31 x 20 mm, ReproSil-Pur C18-AQ, ThermoScientific) and eluted on-line with an analytical RP column (0.075 x 100 mm, ReproSil-Pur C18-AQ, ThermoScientific), operating at 300 nl/min and using a 40-min gradient from 0% to 40% solvent B [solvent A: 0.1% formic acid (v/v), solvent B: 0.1% formic acid (v/v), and 80% acetonitrile (v/v)]. For analysis of PRMT1 labeled with Cl-amidine, a m/z 400-1600 survey scan was performed followed by the fragmentation of the ten most intense ions. A database search with the MS/MS results was performed using the SEQUEST algorithm to identify the modified cysteine residue(s) (m/z 377.15). For analysis of PRMT1 labeled with C21, the LTQ was programmed in the ‘selected MS/MS ion monitoring’ (SMIM) mode. The m/z
400-1600 survey scan was first performed in order to check for the presence of digested peptides as well as peptide separation along the gradient. This survey scan is followed by dependent MS/MS scans that fragment the six most intense ions to confirm the presence of the protein of interest. Next, MS/MS spectra were programmed, focusing on \( m/z \) 564.55, which corresponds to the quadruply-charged precursor ion of the modified KVIGIEC*SSISDYAVK peptide, and on \( m/z \) 752.40, corresponding to the same modified peptide but in its triply-charged state. Note that the quadruple charge stems from a possible charge on the fragmented C21 peptide and that the MS/MS scans were repeated twice.

5.2.4 IC\(_{50}\) Assay

IC\(_{50}\) values were determined using a gel-based radioactive assay with a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 25 \( \mu \)M AcH4-21, 0.2 \( \mu \)M PRMT1, and varying concentrations of inhibitor. Samples were pre-incubated for 10 min at 37 °C and the reaction was then initiated with 15 \( \mu \)M \([^{14}\text{C}]-\)labeled SAM. The reaction was quenched with tris-tricine gel loading dye after 15 min and the samples were run on tris-tricine gels. Incorporated radioactivity was measured by phosphorimage analysis (Molecular Dynamics). Each assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within \( \leq 20\% \). The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq. 1,

\[
\text{Fractional activity of PRMT1} = \frac{1}{1 + ([I]/IC_{50})} \tag{1}
\]

in which the concentration of inhibitor equals \([I]\) and the concentration of inhibitor that yields half-maximal activity equals the IC\(_{50}\).

5.2.5 Dialysis Experiments
For C21, a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 125 µM C21, and 10 µM enzyme (WT PRMT1 or C101A mutant) in a final volume of 150 µL was incubated for 1 h at 37 °C. Note that a control reaction was also performed without C21. Samples were then dialyzed for 24 h at 4°C against 100 mM HEPES, 200 mM NaCl, 1 mM DTT, 2 mM EDTA, and 10% glycerol in a total volume of 2 L. An aliquot was removed from all samples, before dialysis, and activity was measured using the gel-based assay described in Chapter 2, with 15 µM 14C-SAM, 25 µM AcH4-21, 125 µM C21, and 0.2 µM enzyme or enzyme-inhibitor complex for 15 min at 37 °C, followed by quenching with tris-tricine gel loading dye. After dialysis, protein concentration in each sample was determined by Bradford assay and activity was measured with 15 µM 14C-SAM, 25 µM AcH4-21, and 0.2 µM enzyme or enzyme-inhibitor complex for 15 min at 37 °C, followed by quenching with tris-tricine gel loading dye. Samples were separated by tris-tricine gels and radioactivity was measured using phosphorimage analysis (Molecular Dynamics). The product formed by the control reactions was set to 100% and the percent activity of the other samples was determined based on this number. The same procedure was repeated for Cl-acetamidino-N-ethyl-aminoadenosine, except the concentration of inhibitor was 1 mM and only the WT enzyme was assayed.

5.2.6 Synthesis of N-ethyl-aminoadenosine

A solution of 100 mg (0.35 mmol) of 6-chloropurine riboside and 70 µL (1.1 mmol) of ethylene diamine in 3 mL of IPA was refluxed for 1 h at 83 °C. Ether was then added to the reaction and the compound was purified by trituration. The ether was then decanted and the product, N-ethyl-aminoadenosine, was dried in vacuo and the mass was
verified by LC-MS: expected = \textit{m/z} 311.14 (M + H\(^{+}\)), observed = \textit{m/z} 311.1 (M + H\(^{+}\)). N-ethyl-aminoadenosine was used in subsequent experiments without further purification.

5.2.7 Synthesis of Cl-acetamidino-N-ethyl-aminoadenosine

A solution of 39 mg (0.126 mmol) of the N-ethyl-aminoadenosine, 30 mg (0.189 mmol) of ethylchloroacetimidate hydrochloride, and 71.7 µL (0.377 mmol) of potassium carbonate in 5 mL of methanol was then rocked overnight at rt. The solvents were evaporated \textit{in vacuo} and the product was purified by RP-HPLC with a linear gradient of water (0.05% TFA)/acetonitrile (0.05% TFA). The mass of the product was verified by LC-MS: expected = \textit{m/z} 386.13 (M + H\(^{+}\)), observed = \textit{m/z} 386.1 (M + H\(^{+}\)).

5.3 Results and Discussion

5.3.1 Identifying the Site of Modification of Haloacetamidine-Based Inactivators

Our lab previously described two irreversible inactivators of PRMT1, \textit{i.e.}, Cl-amidine and C21, with the latter of the two being the most potent PRMT1 inhibitor described to date (Obianyo et al. 2010). Cl-amidine was originally described as a potent PAD inhibitor (Luo et al. 2006a and Luo et al. 2006b) and because both families of enzymes modify arginine residues, it was thought that it might inhibit the PRMTs as well, which proved to be true. The site of modification of the PADs is an active site cysteine residue that is critical for activity (Luo et al. 2006a and Luo et al. 2006b); however, it has been unclear as to the identity of the site of modification for PRMT1, as a cysteine residue is not important for catalysis. Discovery of the site of modification would aid in the development of more potent and selective inhibitors by allowing for modification of the current inhibitors or by creation of new inhibitors.
Figure 5.7 MS/MS of the site of modification of PRMT1 with (A) Cl-amidine and (B) C21. For both inactivators, KVIGIECSSISDYAVK was identified as the modified peptide, with C101 as the modified residue.
Because C21 is peptide based, which can complicate the MS/MS analysis, PRMT1 was first labeled with Cl-amidine and then an in-gel trypsin digest was performed followed by MS/MS analysis. The results show that C101, an active site cysteine residue, is the site of modification (Figure 5.7A). The experiment was then repeated with C21 and the results revealed that C101 is the site of modification for this inactivator as well (Figure 5.7B).

5.3.2 Characterization of the C101A Mutant

To characterize the role of C101 in PRMT1 catalysis, an alanine mutant was made and the kinetic parameters were determined (Tables 5.2 and 5.3). Unlike the modified cysteine in the PADs, mutation of this cysteine residue confirmed previous observations (Zhang et al. 2003) that it does not play a role in substrate binding or catalysis, as demonstrated by the similar \( K_m \), \( k_{cat} \), and \( k_{cat}/K_m \) values. In fact, based on \( k_{cat}/K_m \), it is a

<p>| Table 5.2 Kinetic parameters of PRMT1 mutants for the AcH4-21 peptide |
|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Mutant</th>
<th>( K_m ) (µM)</th>
<th>Fold</th>
<th>( k_{cat} ) (min(^{-1}))</th>
<th>Fold</th>
<th>( k_{cat}/K_m ) (M(^{-1})·min(^{-1}))</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(^a)</td>
<td>1.1 ± 0.5</td>
<td>-----</td>
<td>4.6 x 10(^{-1}) ± 2 x 10(^{-2})</td>
<td>-----</td>
<td>4.1 x 10(^5)</td>
<td>-----</td>
</tr>
<tr>
<td>C101A(^a)</td>
<td>0.8 ± 0.57</td>
<td>0.73</td>
<td>1.03 x 10(^{-1}) ± 4 x 10(^{-2})</td>
<td>0.45</td>
<td>1.3 x 10(^6)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

\(^a\)[SAM] = 15 µM.

<table>
<thead>
<tr>
<th>Table 5.3 Kinetic parameters of PRMT1 mutants for SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>WT(^a)</td>
</tr>
<tr>
<td>C101A(^a)</td>
</tr>
</tbody>
</table>

\(^a\)[AcH4-21] = 100 µM.
slightly better enzyme. We then hypothesized that the C101A mutant would likely not be inhibited by C21, due to the fact that the enzyme can no longer be covalently modified. The results show, however, that the C101A mutant is still inhibited by C21, but the IC\textsubscript{50} value is increased by ~18-fold (Table 5.4). These results suggest that C21 may be a reversible inhibitor for C101A, while an irreversible inactivator of the WT enzyme. Dialysis experiments were performed to confirm this hypothesis and the results

<table>
<thead>
<tr>
<th>Table 5.4 IC\textsubscript{50} values for C21</th>
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</thead>
<tbody>
<tr>
<td>Enzyme</td>
</tr>
<tr>
<td>PRMT1</td>
</tr>
<tr>
<td>C101A</td>
</tr>
</tbody>
</table>

Figure 5.8 Dialysis experiments of WT PRMT1 and the C101A mutant with C21. The WT enzyme remains inactivated after dialysis, thus demonstrating that C21 is an irreversible inactivator towards the enzyme. The C101A mutant regains activity after dialysis, thus showing that C21 is only a reversible inhibitor for the mutant enzyme.
showed that the C101A mutant regained activity after dialysis (Figure 5.8). The WT enzyme, however, remained inactivated, thus suggesting that C21, is a reversible inhibitor for C101A. These results also confirm that C101 is the relevant site of modification.

5.3.3 Design of SAM Analogues as PRMT1 Inactivators

The fact that PRMT1 is modified at C101 by C21 would suggest that the residue is located in the substrate binding pocket. Surprisingly however, examination of the crystal structure shows that C101 faces the SAM binding pocket instead and is only 3.9 Å from the adenine rings and 4.1 Å from the ribose of SAH (Figure 5.9). Based on these structural observations, as well as the fact that SAM analogues (i.e. SAH and sinefungin) are potent inhibitors of the PRMTs, vide supra, we hypothesized that we could create
more potent inactivators for PRMT1 by designing analogues of SAH that contain warheads that react with cysteine (i.e., chloroacetamidine, fluoromethylketone, and acrylamide). As a starting point for inhibitor development, only the adenosine portion of SAH was used for the first SAM analogue, i.e., Cl-acetamidino-N-ethyl-aminoadenosine. The synthetic scheme for this compound can be found in Figure 5.10. Briefly, ethylene diamine was coupled to 6-chloropurine riboside followed by coupling of ethylchloroacetamidate to yield the final product.

Figure 5.10 Synthesis of N-ethyl-aminoadenosine and Cl-acetamidino-N-ethyl-aminoadenosine.

IC$_{50}$ values were determined for adenosine, N-ethyl-aminoadenosine, and Cl-acetamidino-N-ethyl-aminoadenosine and can be shown in comparison to C21 in Table 5.5. The results show that the chloroacetamidine warhead is critical for inhibition, as demonstrated by the large IC$_{50}$ values, i.e., >2000 µM, for adenosine and N-ethylaminoadenosine; in comparison, the IC$_{50}$ of Cl-acetamidino-N-ethyl-aminoadenosine is 200 ± 109 µM. The high value for the C101A mutant, >1000 µM, further suggests that covalent modification of this residue by Cl-acetamidino-N-ethyl-aminoadenosine is critical for inhibition. To confirm that this SAM analogue is an irreversible inhibitor, dialysis experiments were performed and the results show that, although not as potent as
C21 (Figure 5.8), activity is not regained after dialysis and therefore it too is an irreversible inhibitor (Figure 5.11).

### Table 5.5. IC\textsubscript{50} Values for PRMT1 & mutant with inhibitors & inactivators

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Mutant</th>
<th>IC\textsubscript{50} (µM)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-21</td>
<td><img src="image1" alt="Structure" /></td>
<td>WT\textsuperscript{ab}</td>
<td>1.8 ± 0.1</td>
<td>-----</td>
</tr>
<tr>
<td>Cl-acetamidino-N-ethyl-aminoadenosine</td>
<td><img src="image2" alt="Structure" /></td>
<td>C101A\textsuperscript{ab}</td>
<td>32 ± 6</td>
<td>18</td>
</tr>
<tr>
<td>N-ethyl-aminoadenosine</td>
<td><img src="image3" alt="Structure" /></td>
<td>WT\textsuperscript{ab}</td>
<td>200 ± 109</td>
<td>111</td>
</tr>
<tr>
<td>Adenosine</td>
<td><img src="image4" alt="Structure" /></td>
<td>C101A\textsuperscript{ab}</td>
<td>&gt;1000</td>
<td>&gt; 556</td>
</tr>
<tr>
<td>Adenosine</td>
<td><img src="image5" alt="Structure" /></td>
<td>WT\textsuperscript{ab}</td>
<td>&gt;2000</td>
<td>&gt; 1111</td>
</tr>
<tr>
<td>Adenosine</td>
<td><img src="image6" alt="Structure" /></td>
<td>C101A\textsuperscript{ab}</td>
<td>&gt;2000</td>
<td>&gt; 1111</td>
</tr>
</tbody>
</table>

\textsuperscript{a}AcH4-21 = 25 µM; \textsuperscript{b}SAM = 15 µM
Figure 5.11 Dialysis experiments of WT PRMT1 with Cl-acetamidino-N-ethyl-aminoadenosine. The WT enzyme remains inactivated after dialysis, thus demonstrating that Cl-acetamidino-N-ethyl-aminoadenosine is an irreversible inactivator.

5.4 Conclusions

The development of potent, selective, and cell permeable inhibitors of the PRMTs has been a challenge due to the structural similarities between the family members. Our lab has previously described the most potent inactivator to date, C21 (Obianyo et al. 2010), however, the site of modification was a mystery. To identify the site of modification, PRMT1 was first labeled with Cl-amidine, a potent inhibitor for the PADs, and the MS/MS analysis revealed that C101 was modified. The same experiment was performed with C21 and C101 was once again identified as the modified residue. A C101A mutant was created to determine if this residue is important for catalysis, and the kinetic parameters demonstrated that it is not. Although the C101A mutant is still inhibited by C21, dialysis experiments determined that the inhibitor was reversible compared to its irreversibility with the WT enzyme. Interestingly, this residue has
previously been described as a hyper-reactive cysteine residue (Weerapana et al. 2010), thus making it a good target for covalent modification. In fact, examination of the structure of PRMT1 revealed that C101 is in the SAM binding pocket. Therefore, we hypothesized that SAM analogues containing warheads that react with cysteine would be potent inhibitors of PRMT1. To test our hypothesis, a SAM analogue, \textit{i.e.}, Cl-acetamidino-N-ethyl-aminoadenosine, was synthesized. IC$_{50}$ values were determined for not only the inhibitor, but for its precursors, adenosine, and N-ethyl-aminoadenosine, and the results show that the warhead is critical for inhibition. Comparison of the values for the WT enzyme and the C101A mutant suggests that C101 is the site of modification and important for inhibition with the analogue. In addition, dialysis experiments confirmed that Cl-acetamidino-N-ethyl-aminoadenosine is an irreversible inhibitor of the WT enzyme.

Interestingly, a cysteine residue as this position is only found in PRMT1 and PRMT8 (Figure 5.9A). This observation may explain why C21 is selective for PRMT1 over PRMT3 and CARM1, who each contain a cysteine residue that is buried away from the active site (Figure 5.9B-C). It is thus surprising that C21 is also quite potent for PRMT6, even though an active site cysteine residue is not present in the crystal structure. However, a cysteine residue is located on the N-terminal tail of this isozyme, which looks as though it may be in a position to react with C21 after it is bound, based on the crystal structures of other PRMTs.

Of most interest is a cysteine residue in the active site of PRMT5 that is opposite the position of C101 in PRMT1 (Figure 5.9D). Although the reactivity of this cysteine residue is unknown, this observation suggests that it may be possible to develop SAM
analogues that are individually selective for PRMT1 and PRMT5 by varying the position of the warhead.

Figure 5.12 Structural representation of PRMT cysteine residues around the active site for (A) PRMT1, (B) PRMT3, (C) CARM1, and (D) PRMT5. Note that the transparent spheres (B and C) are facing away from the active site. This figure was prepared with UCSF Chimera using the coordinates from PRMT1 (PDBID 1ORI), PRMT3 (PDBID 3SMQ), CARM1 (PDBID 3B3F), and PRMT5 (PDBID 4GQB).

Overall, the results described in this chapter have opened up a new door for our efforts to develop potent and selective inhibitors and inactivators of not only PRMT1, but other isozymes as well. Future studies will include the synthesis of SAM analogues with SAH as the backbone and a variety of different linkers and warheads. We hypothesize that our newly designed inhibitors will be the most potent and selective PRMT inhibitors to date.
CHAPTER 6
Crosstalk Between Methylation and Phosphorylation

6.1 Introduction

As mentioned in Chapter 1, the best characterized examples of crosstalk between two or more different post-translational modifications (PTMs) occur with respect to histones. These examples demonstrate the critical roles that crosstalk plays in regulating cell signaling pathways. Recently, however, non-histone crosstalk has been observed between serine/threonine phosphorylation and the modification of arginine residues within kinase consensus sequences. We hypothesize that crosstalk between serine/threonine phosphorylation and arginine modifications (Figure 6.1) is a general mechanism to regulate eukaryotic cell signaling, given that these neighboring arginine residues are key substrate recognition elements for many protein kinases (Figure 6.2).

Figure 6.1 Potential model for crosstalk between arginine methylation and phosphorylation.

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3 Adapted with permission from Rust, H.L.; Thompson, P.R., Kinase consensus sequences: a breeding ground for crosstalk. ACS chemical biology 6(9), 881-892. Copyright 2013 American Chemical Society.
Figure 6.2 Serine/Threonine Protein Kinase consensus sequences. A number of serine/threonine protein kinases recognize protein sequences that contain positively charged arginine and lysine residues adjacent to the site of phosphorylation. For example, Akt, prefers substrates that have two arginines (or lysines) at positions -3 and -5 with respect to the modification site that are separated by a variant residue. Each box represents one residue’s position and multiple single letter amino acid codes demonstrate variability within that position. Adapted from (Kinexus 2011).

### 6.1.1 Arginine Methylation Blocks Phosphorylation

Four examples of direct, adjacent cis crosstalk (see Chapter 1) have emerged
involving arginine methylation and serine phosphorylation. First, Yamagata et al. demonstrated that FOXO1 (forkhead box O1) is methylated \emph{in vitro} and \emph{in vivo} by PRMT1 (Protein Arginine Methyltransferase 1) (Yamagata et al. 2008). FOXO1 is a member of the FOXO family of transcription factors, which play essential roles in cell cycle regulation, apoptosis, the oxidative stress response, and overall cell survival (Greer et al. 2005). The sites of FOXO1 modification, R248 and R250, are conserved across family members and species. Interestingly, the FOXO family members are phosphorylated by Akt at three conserved residues, one of which, i.e., S253 in FOXO1 (Brunet et al. 1999), lies adjacent to R248 and R250 (mouse numbering). The authors then demonstrated that methylation of these two arginine residues inhibited phosphorylation of S253, a clear example of \emph{cis} crosstalk. However, the converse was not observed – phosphorylation does not prevent methylation (Yamagata et al. 2008).

Functionally, methylation of R248 and R250 blocks the phosphorylation of S253, thereby preventing the phosphorylation dependent nuclear export of FOXO1 (Zhang et al. 2002). Additional experiments indicated that it is the lack of phosphorylation, and not the presence of methylated arginine residues, that is responsible for inhibiting FOXO1 export (Yamagata et al. 2008). Because the phosphorylation and nuclear export of FOXO1 is associated with its polyubiquitination and degradation by the proteasome (Matsuzaki et al. 2003 and Huang et al. 2005), the authors tested the effects of PRMT1 knockdown on FOXO ubiquitination and stability. The results indicated that PRMT1 knockdown enhanced the polyubiquitination of FOXO1, which promoted its degradation by the proteasome (Yamagata et al. 2008).
Since it is well established that FOXO family members control the response to oxidative stress (Greer et al. 2005), the authors further hypothesized that the methylation of FOXO1 would affect this pathway. As expected, hydrogen peroxide led to an increase in the PRMT1-dependent methylation of FOXO1, which in turn blocked the phosphorylation of S253, and nuclear export. As a result, the transcription of a number of FOXO1-dependent genes was increased including, BIM (BCL-2-interacting mediator), an apoptosis inducing protein (Yamagata et al. 2008). Consistent with this model, when either PRMT1 or FOXO1 were knocked down by siRNA, no increase in BIM transcription was observed. Although PRMT1 knockdown inhibited apoptosis in response to oxidative stress, inhibition of PI3K-Akt signaling has the reverse effect (Yamagata et al. 2008). Taken together, these results demonstrate a functional crosstalk between the methylation of R248 and R250 and phosphorylation of S253 of FOXO1. This mechanism of crosstalk appears to be evolutionarily conserved because methylation of the FOXO1 orthologue in C. elegans, DAF-16, also inhibits its phosphorylation by Akt. Interestingly, this activity in C. elegans appears to play a role in life span extension, as PRMT1 knockouts died significantly earlier than wild type worms, thereby suggesting that PRMT1 inhibition may exert pleiotropic off target effects (Takahashi et al. 2011).

Recently, Sakamaki et al. published a second example of cis crosstalk between PRMT1 and Akt. Specifically, the authors investigated whether the phosphorylation of other proteins that contain an Akt consensus sequence (i.e., RXRXXS/T) are modulated by the methylation of adjacent arginine residues (Sakamaki et al. 2011). Several known Akt substrates (i.e., BAD, PGC-1α, eNOS, p27, GSK3β, and MDM2) were tested as PRMT1 substrates. Only PGC-1α (peroxisome proliferator-activated receptor-γ
coactivator) (Yamagata et al. 2008) and BAD (BCL-2 antagonist of cell death) (Sakamaki et al. 2011), were shown to be methylated by PRMT1. The fact that eNOS, p27, GSK3β, and MDM2 were not methylated (Sakamaki et al. 2011) suggests that additional PRMT1 recognition elements are required for substrate methylation (vide infra). In BAD, the sites of modification were identified as R94 and R96, and, as was the case with FOXO1 (Yamagata et al. 2008), arginine methylation prevented phosphorylation of an adjacent serine residue (i.e., S99), but prior phosphorylation did not affect BAD methylation by PRMT1 (Sakamaki et al. 2011).

Due to the functional role of a methylation/phosphorylation switch in regulating FOXO1 activity (Yamagata et al. 2008), it was probable that crosstalk would also affect the physiological activity of BAD. BAD is a pro-apoptotic member of the BCL-2 protein family and plays a major role in regulating cellular apoptosis (Danial et al. 2008). Previous studies have demonstrated that several kinases and phosphatases are responsible for altering the phosphorylation state of BAD and thus dictating its location and activity (Danial et al. 2008, Yang et al. 1995, Datta et al. 1997, Harada et al. 1999, Bonni et al. 1999, Datta et al. 2000, Tan et al. 2000, Virdee et al. 2000, Ayllon et al. 2000, Chiang et al. 2001, Wang et al. 1999). For example, when BAD is dephosphorylated, it binds to the pro-survival proteins BCL-XL/BCL-2 and displaces the pro-apoptotic proteins BAK and/or BAX (from BCL-XL/BCL-2) to create activated homodimers that form a pore in the mitochondria, which ultimately leads to apoptosis (Danial et al. 2008, Yang et al. 1995, Dewson et al. 2009). However, in response to cellular stress, BAD is phosphorylated at S75, S99, and S118. Once phosphorylated, BAD binds to a 14-3-3 protein and is subsequently removed from the mitochondria and sequestered in the
As a consequence, the pro-apoptotic function of BAD is muted (Datta et al. 1997, Harada et al. 1999, Bonni et al. 1999, Datta et al. 2000, Tan et al. 2000, Virdee et al. 2000, Zha et al. 1996, del Peso et al. 1997). Based on this model, one would expect that decreased methylation of BAD by PRMT1 would increase phospho-BAD levels, which would lead to enhanced 14-3-3 binding, sequestration in the cytoplasm, decreased caspase activity, and consequently an increase in cell viability, all of which were observed when PRMT1 was knocked down by siRNA (Sakamaki et al. 2011). In contrast to the situation with FOXO1, methylation of BAD was not triggered by oxidative stress or known BAD activators. Thus it is unclear whether BAD methylation is constitutive or occurs in response to an unknown stimulant (Sakamaki et al. 2011). In any case, these observations demonstrate that the methyltransferase activity of PRMT1 is critical for the pro-apoptotic function of BAD through its prevention of Akt mediated phosphorylation of S99 (Sakamaki et al. 2011).

The previous two examples involved PRMT1, a Type I PRMT that catalyzes the asymmetric dimethylation of arginine residues in proteins. In the third example of direct adjacent cis crosstalk, PRMT5, a Type II PRMT that symmetrically dimethylates arginines, is the responsible enzyme. Specifically, Guo et al. show that FEN1 (flap endonuclease 1) is methylated at R192 by PRMT5 and that methylation of this particular arginine inhibits the phosphorylation of S187 by Cdk2-cyclin E (cyclin dependent kinase-2). As with the previous examples, the reverse scenario was not observed, i.e., phosphorylation does not prevent methylation (Guo et al. 2010). Since FEN1 phosphorylation prevents its binding to PCNA (proliferating cell nuclear antigen) (Henneke et al. 2003), it is unsurprising that by preventing phosphorylation, methylation
promotes the PCNA and FEN1 interaction. However, methylation occurring after phosphorylation did not re-establish this protein complex, confirming that the inhibition of phosphorylation, and not methylation alone, is responsible for the observed effect (Guo et al. 2010). The interaction between PCNA and FEN1 is responsible for localizing FEN1 to the site of replication. This interaction is important because FEN1 is an exo- and endonuclease involved in essential DNA processes, such as replication and repair (Shen et al. 2005). A R192K mutant, which cannot be methylated, abrogated both the PCNA/FEN1 interaction and the localization of FEN1 to the site of replication. As a consequence, a buildup of DNA double-stranded breaks was detected, followed by slower progression through the cell cycle, and ultimately mitotic arrest (Guo et al. 2010). Because other studies have shown that PRMT1 plays a role in the oxidative stress response (Yamagata et al. 2008), the authors also investigated the effect of hydrogen peroxide on the methylation of FEN1. The results showed that oxidative stress results in localization of methylated FEN1 to the nucleus. FEN1 that lacked methylation resulted in a decrease in cell survival and an increase in mutations, thus demonstrating a correlation between arginine methylation and DNA repair (Guo et al. 2010). These observations demonstrate that PRMT5 dependent methylation of the R192 residue of FEN1 plays a critical role in preventing Cdk2-cyclinE dependent phosphorylation of S187 and subsequently allows for proper DNA replication and repair. With respect to human disease, these results suggest that PRMT5 inhibition would synergize with DNA damaging agents as a way to treat cancer.

6.1.2 Phosphorylation Blocks Arginine Methylation

The above crosstalk examples demonstrate a functional role for the inhibition of
serine phosphorylation by arginine methylation, but not the inverse. More recently, Sims et al. uncovered such an example of direct adjacent cis crosstalk, where phosphorylation of RNA polymerase II (RNAPII) prevents its methylation at R1810 (Sims et al. 2011). The CTD (carboxy terminal domain) of RNAPII contains a series of heptad repeats whose consensus sequence is YSPTSPS (Egloff et al. 2008), however, several of these repeats contain arginine or lysine substitutions in the last position (i.e., YSPTSP[R/K]) (Sims et al. 2011). It is known that S2 and S5 of these sequences can be phosphorylated by P-TEFb (positive transcription elongation factor b) and CAK, (CDK activating kinase), respectively (Sims et al. 2011). Phosphorylation of these residues activates RNAPII and aids in the recruitment of essential proteins (Fong et al. 2001 & Misteli et al. 1999) that are important for gene transcription (Egloff et al. 2008). Due to the unique nature of the arginine and lysine substitutions, the authors investigated whether they were specifically modified. The only PTM to be identified was methylation of R1810 by CARM1 (co-activator-associated protein arginine methyltransferases 1) or PRMT4. Interestingly, phosphorylation of S2 and S5 prevented methylation of R1810 but methylation did not prevent phosphorylation. Also, the presence of both methylation and phosphorylation was observed in vivo suggesting that methylation occurs before phosphorylation (Sims et al. 2011). The functional consequence of a lack of methylation of R1810 is downregulated transcription of small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) (Sims et al. 2011).

6.1.3 Distal Crosstalk Between Arginine Methylation and Phosphorylation

Although the main focus of this chapter is direct adjacent cis crosstalk within kinase consensus sequences, it is worth noting two additional examples involving distal
non-histone crosstalk between arginine methylation and phosphorylation. The first example involves an interesting interplay between the CARM1 dependent methylation of R3 in C/EBPβ (CCAAT/enhancer-binding protein β) (Kowenz-Leutz et al. 2010), a transcription factor, and the phosphorylation of T253 by MAPK (Nakajima et al. 1993). Here, phosphorylation of T253 abrogates the interaction between C/EBPβ and CARM1, which abolishes the methylation of R3 (Kowenz-Leutz et al. 2010). Unmethylated C/EBPβ is then free to bind to the SWI/SNF nucleosome remodeling and the Mediator transcriptional co-activator complexes to facilitate the increased transcription of C/EBPβ-dependent genes. Thus, R3 methylation inhibits interactions between C/EBPβ, SWI/SNF, and Mediator, and as a consequence, down regulates the transcription of genes under the control of C/EBPβ.

A second example of distal crosstalk involves the members of the STAT (signal transducer and activator of transcription) family of proteins, which play important roles in cell differentiation, survival, and apoptosis (Calo et al. 2003). Given that STAT1 is methylated by PRMT1 at R31, a conserved arginine residue (Mowen et al. 2001), Chen et al. investigated whether the corresponding arginine in STAT6, R27, was also modified, and found that this was the case. However, the responsible methyltransferase was not identified (Chen et al. 2004). Nevertheless, the authors did show that the lack of methylation prevented phosphorylation of a distal tyrosine residue (i.e., Y641) and consequently inhibited nuclear translocation, abrogated DNA binding, and decreased protein stability. It was shown that this crosstalk was not due to the activation of tyrosine phosphatases. Overall, the results demonstrated that arginine methylation of STAT6 is required for phosphorylation of an essential tyrosine residue, which ultimately effects the
location of the protein, its DNA binding capabilities, and protein stability (Chen et al. 2004).

6.1.4 Structural Basis for Crosstalk

Protein kinases typically bind and phosphorylate serine, threonine, or tyrosine residues within a distinct consensus sequence. Of these kinases, Akt is a perfect example of how PTMs within a consensus sequence can alter substrate binding. For Akt, the consensus sequence (i.e., RXR[S/T/A][S/T/A][S/T][F/L]) contains two positively charged arginine residues at the -3 and -5 positions relative to the phosphorylation site.

![Figure 6.3 Structural basis for crosstalk. A structure of Akt (white) bound to a GSK3β (cyan) derived peptide demonstrates that arginine residues in the -5 and -3 positions are critical for Akt substrate recognition. R-5 forms direct and indirect hydrogen bonds with several key residues (i.e. E279, Y316, E342), as well as, with T-2 on the peptide. This residue is also capable of forming a salt bridge with E279. R-3 forms both a hydrogen bond and salt bride with E236. The methylation of both R-5 and R-3 would disrupt these key interactions and thus result in the observed inhibition of serine phosphorylation, which is demonstrated in several examples presented in this review (i.e., FOXO1 (Yamagata et al. 2008) and BAD (Sakamaki et al. 2011). This figure was prepared with UCSF Chimera using the coordinates for the Akt•GSK3β peptide complex (PDBID 1O6L).]
Based on the structure of Akt bound to a peptide whose sequence is derived from GSK3β (Yang et al. 2002) (Figure 6.3), it is apparent that R-5 forms several direct and indirect hydrogen bonds with surrounding Akt residues, including E279, Y316, and E342, as well as T-2 on the peptide itself. In addition, the positive charge of R-5 and the negative charge of E279 are likely capable of forming a salt bridge. R-3 also forms a hydrogen bond and salt bridge with E236, and, although the distance between R-3 and D440 is too great for a hydrogen bond interaction, it is also possible that there are electrostatic attractions between these two residues. In the examples described above, both R-3 and R-5 were methylated and this prevented the phosphorylation of the targeted serine residue (Yamagata et al. 2008 & Sakamaki et al. 2011). The structural basis for this crosstalk is easily discerned. For example, while the formation of an asymmetrically dimethylated arginine would not alter the charge of the residue, it would undoubtedly create steric bulk. This added steric bulk would prevent the formation of key hydrogen bonds, with for example E279 and Y316 in the substrate binding cleft, which would result in an inability to properly bind the substrate and thereby inhibit phosphorylation. Similar effects would be expected for the methylation of R-3. One could imagine that deimination of these two arginine residues by the PADs would likely yield the same result. However, instead of adding steric bulk, abrogation of protein binding would be due to the neutralization of the positively charged arginine residue and the disruption of proper hydrogen bonding. Although the conversion to citrulline would still allow for a lower degree of hydrogen bond formation, the carbonyl oxygen would only be a
hydrogen bond acceptor, thus terminating the bidentate interactions between R-3 and E236 and R-5 and E279.

Interestingly, phosphorylation does not block methylation by PRMT1 and PRMT5. Based on the crystal structure of PRMT1 (Zhang et al. 2003), as well as work from our own lab (Osborne et al. 2007), this observation is easily rationalized. For example, while the surface of PRMT1 is highly negatively charged (Zhang et al. 2003), which would suggest that the introduction of a phosphate group would lead to electrostatic repulsions, we have shown that the residues between the site of methylation and distal positively charged residues are relatively unimportant for substrate recognition (Osborne et al. 2007). Although a similar explanation is likely for PRMT5, detailed substrate specificity studies have not been performed on this isozyme. In contrast to the situation with PRMTs 1 and 5, phosphorylation of S2 and S5 in RNAPII blocked the methylation of R1810 by CARM1 (Sims et al. 2011). Given that detailed substrate specificity studies have also not been performed for this enzyme and no structures of CARM1 bound to cognate peptide substrates are available, it is difficult to speculate on why phosphorylation blocks methylation. Nevertheless, the addition of two phosphate groups adjacent to the site of methylation would likely not only cause a perturbation in the peptide structure itself, due to repulsion between the two phosphate groups, but would also likely disrupt key interactions within the substrate binding cleft.

6.1.5 Role of Additional Modifications

Although the discussion so far has focused mainly on arginine methylation, this residue is also subject to deamination and it is likely that this additional modification will exhibit crosstalk with protein phosphorylation. In support of this notion, the Coonrod
group recently showed that Elk-1 (ETS like gene 1), a member of the ETS family of transcription factors, is deiminated by PAD4 and that this modification increases Elk-1 phosphorylation by ERK2. Although it has yet to be established that these two modifications occur in the same consensus sequence, it is known that phosphorylation facilitates a tight interaction between Elk-1 and p300 leading to increased histone acetylation and ultimately the activation of c-Fos (Zhang et al. 2011).

One final layer of complexity is the role of antagonistic PTMs. For example, we and others have shown that deimination/citrullination of an arginine residue can antagonize/prevent the methylation of that same arginine residue (Hidaka et al. 2005, Kearney et al. 2005, Thompson et al. 2006, Raijmakers et al. 2007, Cuthbert et al. 2004, Guo et al. 2011). In addition, serine O-GlcNAcylation has been shown to antagonize phosphorylation of the same serine residue (Hart et al. 2010 & Wang et al. 2010). Overall, these individual observations suggest that crosstalk within kinase consensus sequences is potentially quite complex, has multiple levels, and that eukaryotic cell signaling is not well represented by the linear pathways often depicted in textbooks.

6.1.6 Crosstalk and Disease

Crosstalk between protein phosphorylation and modification of arginine residues within kinase consensus sequences potentially has significant relevance to human disease. This is the case because the modification of these basic residues can potentially have either growth promoting or growth suppressing effects. For example, in the two examples of Akt crosstalk, PRMT1 opposes the effects of Akt-mediated phosphorylation (Yamagata et al. 2008 & Sakamaki et al. 2011). Given that PI3K-Akt signaling is overactive in multiple cancers, these results suggest that the inhibition of PRMT1 would
further stimulate the growth promoting and cell survival effects of Akt signaling. Nevertheless, PRMT1 has been shown to be required for the growth promoting effects of estrogen signaling and siRNA knockdown of PRMT1 has been shown to suppress the growth of MCF7 cells (Le Romancer et al. 2008). As such, it is unclear whether PRMT1 represents a valid target for the development of an anticancer therapeutic, thereby highlighting the critical need for developing bioavailable PRMT1 inhibitors that can be used to specifically address this question. Additionally, the putative role of arginine modifying enzymes in regulating kinase signaling highlight the possibility that the effects of inhibitors targeting these enzymes may be due not only to effects on gene transcription but also to effects on kinase signaling pathways. Again, this highlights the need for additional research to examine the links between consensus crosstalk and human disease. Finally, the fact that the mutation of K303 to an arginine residue in ERα is present in one-third of patients with premalignant hyperplasias (Fuqua et al. 2000) is highly interesting because it suggest that cancer associated mutations can impinge on crosstalk. Given the numerous PRMTs, PADs, and kinases, research in this area is undoubtedly an untapped treasure waiting to be discovered.

6.1.7 Predicting Crosstalk

Given that protein kinase substrates can be readily predicted based on the presence/absence of a particular consensus sequence, the most obvious question is whether it is possible to predict crosstalk. The answer appears to be yes. Below we predict potential crosstalk between Akt substrates and PRMT1-mediated methylation. Note that we focused on these two enzymes because of prior precedents with Akt (see above) and our own expertise in predicting PRMT1 substrates (Osborne et al. 2007).
Additionally, PRMT1 is responsible for 85% of all PRMT activity *in vivo* (Tang et al. 2000 & Nicholson et al. 2009), thus it is likely that, if kinase consensus crosstalk is a global mechanism for cellular regulation, PRMT1 would be the principal isozyme involved. Nevertheless, it should be recognized that the same approach could be taken to predict crosstalk between any given kinase with a known consensus sequence and a lysine or arginine modifying enzyme whose substrate specificity determinants are known.

In contrast to kinases, the distinct substrate recognition sequences for PRMT1, and the PRMTs in general, are relatively unknown. In an effort to determine a minimal peptide substrate, based on the N-terminus of histone H4, our lab discovered that positively charged residues distal to the site of methylation are important for substrate recognition and catalysis (Osborne et al. 2007). This makes sense because the surface of PRMT1 is negatively charged and therefore electrostatic interactions between the protein and the substrate are likely present (Zhang et al. 2003 & Osborne et al. 2007). With this knowledge, we investigated whether similarities are present between histone H4 and the recently discovered PRMT1 substrates described above. As shown in Table 6.1, for histone H4, BAD, and the FOXO family members, two arginine residues, in close proximity to each other, are present distal to the site of methylation in addition to a number of other positively charged residues. This observation coincides with our previous findings (Osborne et al. 2007). Closer examination of Akt substrates that failed to be methylated by the PRMTs, (e.g., eNOS, p27, and GSK3β) (Sakamaki et al. 2011) shows that they lack distal positively charged residues throughout the intervening sequences (Table 6.1). The one exception is MDM2. However in this case, the presence of several negatively charged glutamates likely masks the presence of distal positively charged residues.
In light of this analysis, we hypothesized that novel Akt/PRMT1 substrates can be predicted based on the identity of residues downstream from the kinase consensus sequences (Table 6.2). Note that we have stratified the Akt substrates into three groups, i.e., highly probable, likely, and unlikely PRMT1 substrates. Highly probable substrates were selected based on the presence of at least two distal arginine residues separated by one or two variable residue(s), as these RXR and RXXR motifs are a common theme among the known PRMT substrates. These predictions also include at least one other distal positively charged residue in addition to these motifs. Likely substrates include those Akt substrates that possess a number of positively charged residues distal from the predicted sites of methylation, but do not possess the RXR or RXXR motifs, or do not contain an additional positively charged residue. Note that further studies will need to be conducted to determine whether lysine can substitute for arginine in these positions or if the presence of several nonspecific positively charged residues alone is enough for efficient substrate recognition and catalysis. The effect of

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H4</td>
<td>3-RGKGGKGLGKGGAKKRHKVLR-24</td>
<td>transcriptional regulation</td>
</tr>
<tr>
<td>BAD</td>
<td>94-RCRRSAPNLWAAQRYGSRSLRM-117</td>
<td>pro-apoptotic</td>
</tr>
<tr>
<td>FOXO1</td>
<td>251-RRRAASMNDNNSKFAKSRRAAKK-274</td>
<td>transcription (pro-apoptotic)</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>566-RCRRSRFSRHRCSRSPYRSRSR-589</td>
<td>gluconeogenesis</td>
</tr>
<tr>
<td>e-NOS</td>
<td>1172-RIRTQSFSLQERQLRGAVPWAEP-1195</td>
<td>vasodilation, angiogenesis</td>
</tr>
<tr>
<td>GSK3-β</td>
<td>127-RPRTTSFAESCKPVQOPSFGSMK-27</td>
<td>cell cycle, apoptosis</td>
</tr>
<tr>
<td>p27</td>
<td>152-RKRPATDSTQKNRANRTSENV-175</td>
<td>cell cycle</td>
</tr>
<tr>
<td>MDM2</td>
<td>161-RRRAISETENSDLGKRQRKH-184</td>
<td>proteolysis</td>
</tr>
<tr>
<td>MDM2</td>
<td>181-RKRRHKSDSISLSPFDESLALC-204</td>
<td>proteolysis</td>
</tr>
</tbody>
</table>

a Known PRMT1 substrate. b Not known to be an Akt Substrate. c Numbers correspond to human FOXO1. Mouse FOXO1 begins at 248. d Sites of methylation have not been identified. e Not a PRMT1 Substrate. The light blue represents sites of methylation. The red represents known sites of Akt phosphorylation. The purple represents positively charged residues. The orange represents negatively charged residues.
## Table 6.2. PRMT1 and Akt Crosstalk Predictions

### A. Known PRMT1 & Akt Substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3-RKKGKRKLGGKGA--------KRHRKVRD-24</td>
<td>transcriptional regulation</td>
</tr>
<tr>
<td>BAD&lt;sup&gt;8&lt;/sup&gt;</td>
<td>94-RRGRSRSPNLWAAQ-------RYGRELRMM-117</td>
<td>pro-apoptotic</td>
</tr>
<tr>
<td>FOXO1α&lt;sup&gt;8&lt;/sup&gt;</td>
<td>251-RRRAASDMNSKHF-------KSRRGAAK-274</td>
<td>transcription (pro-apoptotic)</td>
</tr>
<tr>
<td>FOXO3α&lt;sup&gt;8&lt;/sup&gt;</td>
<td>248-RRRAASMDNSKHY-------KSSRGAAK-271</td>
<td>transcription (pro-apoptotic)</td>
</tr>
<tr>
<td>FOXO4&lt;sup&gt;8&lt;/sup&gt;</td>
<td>192-RRRAASMDSSKLL-------RGRKAPKK-215</td>
<td>transcription (pro-apoptotic)</td>
</tr>
<tr>
<td>PGC-1α&lt;sup&gt;5c&lt;/sup&gt;</td>
<td>566-RSRSRFSRHSRSCS--------RSPYRSRSR-589</td>
<td>gluconeogenesis</td>
</tr>
</tbody>
</table>

### B. Highly Probable PRMT1 Substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinus</td>
<td>1175-RSRSRSD-----------RRRKERAKSKEKL-1198</td>
<td>apoptosis</td>
</tr>
<tr>
<td>B-Raf</td>
<td>424-RKRSRSSSED--------RNRKCTGLGRSD-447</td>
<td>Erk1/2 pathway</td>
</tr>
<tr>
<td>Cot (Tpi2)</td>
<td>397-RQQLSALLE--------RKRLLSREKL-419</td>
<td>oncogene</td>
</tr>
<tr>
<td>elf4B</td>
<td>417-RSTSGSSQGTSTTSS-RNARRR-440</td>
<td>translation initiation</td>
</tr>
<tr>
<td>EZH2</td>
<td>16-RKIKVSEYM-----------RLQLKRFRADEV-39</td>
<td>methyltransferases</td>
</tr>
<tr>
<td>FOXG1</td>
<td>274-RRSTSRRAKALF--------KGGALTSST-107</td>
<td>transcription</td>
</tr>
<tr>
<td>HMOX1</td>
<td>183-RSRMNSLMTPAV--------RQUVEQAKT-206</td>
<td>heme oxygenase</td>
</tr>
<tr>
<td>PFKFB2</td>
<td>469-RRNFTSPPLSSNTI--------RRPRNYSV-484</td>
<td>glycolysis</td>
</tr>
<tr>
<td>TERT</td>
<td>222-RRRAASNRSLPLP--------RKRPAAGA-245</td>
<td>telomerase reverse transcriptase</td>
</tr>
<tr>
<td>Tuberin</td>
<td>934-RASTLSNLERPRLS--------RIARPQKGL-957</td>
<td>tumor suppressor</td>
</tr>
<tr>
<td>Tuberin</td>
<td>1457-RPRGYLSAPS--------RRKVRVERD-1480</td>
<td>tumor suppressor</td>
</tr>
</tbody>
</table>

### C. Likely PRMT1 Substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arfaptin</td>
<td>255-RGLSESQAQTFQA-------RKYKLRGD-278</td>
<td>cell survival</td>
</tr>
<tr>
<td>Ataxin</td>
<td>771-RKRRESAPES--------RKLKEKSEDEPLL-794</td>
<td>neurodegeneration</td>
</tr>
<tr>
<td>CK1-D</td>
<td>365-RKRVSM-----------RLHRGAPVNISSSD-388</td>
<td>circadian clock</td>
</tr>
<tr>
<td>GATA-1</td>
<td>305-RNKTSAG--------KGGKRRGGSLG-328</td>
<td>transcription factor</td>
</tr>
<tr>
<td>GATA-2</td>
<td>396-RNRMSN-----------KSKKSGKACFE-419</td>
<td>transcription factor</td>
</tr>
<tr>
<td>p21</td>
<td>140-RKRRQRTMTDFYHS--------KRRILFSKR-163</td>
<td>cell cycle</td>
</tr>
<tr>
<td>PDE3A</td>
<td>288-RRRRSSSVSSASEMSFCSS-RKSHRT-311</td>
<td>regulates cAMP and cGMP</td>
</tr>
<tr>
<td>PRAS40</td>
<td>241-RPRLNTSDFQ--------KLRRKY-256</td>
<td>insulin signaling</td>
</tr>
<tr>
<td>Skp2</td>
<td>67-RKRLKSHGDODDFIV--------RRPKNLRE-90</td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td>TBC1D4</td>
<td>637-RRRATFSPHCST--------KRRKQQRGR-660</td>
<td>Glut4 trafficking</td>
</tr>
<tr>
<td>Tuberin</td>
<td>976-RCRSISVEHV--------RGIQSITLTA-999</td>
<td>tumor suppressor</td>
</tr>
</tbody>
</table>

### D. Unlikely PRMT1 Substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>208-RRRAASGAPTSKCDYNLGTSTIS-231</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>AR</td>
<td>786-RMRHLQEFGLQITPOEFLCMKA-809</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>BIM</td>
<td>84-RRSSLLRSSSSGYESFDSPTRSP-105</td>
<td>pro-apoptotic</td>
</tr>
<tr>
<td>IRS-1</td>
<td>522-RKRTSAGTSPTHQTPSQQSV-545</td>
<td>insulin receptor signaling</td>
</tr>
<tr>
<td>mTOR</td>
<td>2443-RTRRTDSYSAQSVSVEILDGVELE-2466</td>
<td>cell growth</td>
</tr>
<tr>
<td>Nur77</td>
<td>346-RGRLPSKPKQPDDSPANSLPTS-369</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>p300</td>
<td>1829-RRRMASONRGTGVQQGQLPSTP-1852</td>
<td>transcriptional coactivator</td>
</tr>
<tr>
<td>Rac1</td>
<td>66-LRPLPYPQYTVLICVLISVPSAS-89</td>
<td>Rho-GTPase</td>
</tr>
</tbody>
</table>

<sup>6</sup>Known PRMT1 substrate. <sup>8</sup>Not known to be an Akt Substrate. <sup>5c</sup>Numbers correspond to human FOXO1. Mouse FOXO1 begins at 248. <sup>6</sup>Sites of methylation have not been identified. <sup>8</sup>Not a PRMT1 Substrate. The light blue represents sites of methylation<sup>6c</sup>. The red represents known sites of Akt phosphorylation. The purple represents positively charged residues. The orange represents negatively charged residues.

The presence and position of negatively charged residues on substrate recognition also will need further investigation because, as mentioned above, they likely mask the
positively charged residues. Validation of the predicted PRMT1 and Akt substrates would indicate that consensus crosstalk is a general mechanism to control eukaryotic cell signaling.

6.1.8 Research Overview

Herein we describe our initial efforts toward proving that crosstalk between arginine methylation and phosphorylation of serine/threonine residues exists and is possible to predict. Although studies by other groups have already demonstrated crosstalk between PRMT1 and Akt in regards to FOXO1 (see Section 6.1.1), our results with this model will be used as a proof of concept before moving on to our predicted models (i.e., EZH22 & B-Raf). The determination of a minimal peptide substrate for FOXO1, the effect of serine phosphorylation on substrate recognition by PRMT1, processivity experiments, as well as initial efforts to investigate crosstalk between phosphorylation and deminination will be described.

6.2 Materials and Methods

6.2.1 Chemicals

Sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (TRIS), tetramethylethlenediamine, acrylamide, and ammonium persulfate were purchased from Bio-Rad (Hercules, CA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tricine, dithiothreitol (DTT) were purchased from RPI (Mt. Prospect, IL). Acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride and dimethylformamide (DMF) were purchased from Alfa Aesar (Ward Hill, MA). Piperidine was purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-protected amino acids, (ethylenedinitrilo)tetraacetic acid (EDTA), and trifluoroacetic acid (TFA) were
purchased from EMD (Gibbstown, NJ). \(^{14}\)C-labeled SAM was purchased from Perkin-Elmer and \(^{14}\)C-labeled BSA from Sigma-Aldrich. The purification of PRMT1 and the synthesis of the AcH4-21, FOXO1-21, FOXO1-22, and FOXO-30 peptides are outlined in Chapter 2.

### 6.2.2 Purification of PAD4

The purification of PAD4 has been described (Knuckley et al. 2007). In brief, a pGEX-6P-1 construct (Nakashima et al. 1999) containing the PAD4 gene and an N-terminal GST tag was transformed into *E. coli* Rosetta cells. One colony was used to inoculate 5 mL starter cultures containing 50 μg/mL ampicillin and 20 μg/mL chloramphenicol and incubated overnight with shaking at 37 °C. One liter of TB media was inoculated with 10 mL of overnight culture containing 50 μg/mL ampicillin and 20 μg/mL chloramphenicol and grown at 37 °C and 250 rpm until OD\(_{600}\) = 0.8-1.0. Protein expression was then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside and the cells were incubated with shaking at 16 °C overnight. The next day the cells were harvested by centrifugation at 5000 rpm (4400 g) for 10 min. The pellet was resuspended in 30 mL of Lysis Buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 20% glycerol) plus 2 mL of protease inhibitor cocktail (Sigma-P8465). Cells were mixed slowly for 30 min at 4 °C before being lysed by sonication (13 cycles of 15 sec burst with 1 min rest at output of 10 and a constant duty cycle). The lysate was centrifuged at 14,000 rpm (21,808 g) for 30 min and the supernatant was applied to a Glutathione Sepharose 4B (GE Healthcare) column. The column was washed with 50 mL of a low salt buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM DTT, 250 mM NaCl, and 10% glycerol), 50 mL of a high salt buffer (20 mM Tris-HCl pH 8, 1 mM
EDTA, 1 mM DTT, 500 mM NaCl, and 10% glycerol), and eluted with two 25 mL washes of glutathione buffer (50 mM Tris-HCl pH 8.0, 1 mM DTT, and 10 mM reduced glutathione pH 8.0). Fractions were screened on a 12% SDS-PAGE gel and 10 µL of Prescission Protease (GE Healthcare) was added to fractions containing protein followed by dialysis overnight in 20 mM Tris-HCl pH 7.6, 1 mM EDTA, 2 mM DTT, and 500 mM NaCl. Dialysis buffer was exchanged 3x to remove excess glutathione. The next day the protein was re-applied to the Glutathione Sepharose 4B column and collected followed by a 50 mL column wash of glutathione buffer (see above). The flow through was dialyzed against 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, and 2 mM DTT for 3 h. The dialyzed protein was additionally purified by FPLC using a HiTrap Q Fast Flow column (GE Healthcare) with 20 mM Tris-HCl pH 8.5 and 2 mM DTT as buffer A and a linear gradient from 0-100% of buffer B (20 mM Tris-HCl pH 8.5, 2 mM DTT, and 1 M NaCl). Fractions were screened on a 12% SDS-PAGE gel and by a COLDER activity assay. Fractions containing pure and active protein were dialyzed overnight in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 mM DTT, 500 mM NaCl, and 10% glycerol. The next day the protein was concentrated using a 10 kDa Amicon Centriplus centrifugal filter and the concentration was determined using a Bradford assay. The enzyme was flash frozen in liquid nitrogen and stored at -80 °C.

6.2.3 Gel-Based Activity Assay

A gel-based activity assay was used to acquire the steady state kinetic parameters of PRMT1 and has been previously described. (Osborne et al. 2007). Assays were performed in a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 15 µM [14C]-labeled SAM, and a varying concentration of peptide
(0-1000 μM final). Reactions were pre-incubated at 37 °C for 10 min before the addition of PRMT1. Assays with AcH4-21 were quenched after 15 min and assays with the FOXO1 peptides were quenched after 1 h. Each assay was done in duplicate and the standard deviation of the duplicate raw data values agreed within ≤ 20%. The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq 1,

\[ v = \frac{V_{\text{max}}[S]}{(K_m+[S])} \]  

(1)

6.2.4 COLDER Based Activity Assay

Reaction mixtures of 100 mM Tris-HCl pH 7.6, 10 mM CaCl₂, 50 mM NaCl, 2 mM dithiothreitol, and a varying concentrations of benzoyl arginine ethyl ester (BAEE) (0-10 mM) or peptide substrates (0-1mM final) were pre-incubated at 37 °C for 10 min. The reactions were initiated by the addition of PAD4 (0.2 μM final), giving a total volume of 60 μL. Reactions were incubated for 6 min at 37 °C before being quenched by liquid nitrogen. Color development was accomplished by the addition of 200 μL of COLDER Solution (2.25 M H₃PO₄, 4.5 M H₂SO₄, 1.5 mM NH₄Fe(SO₄), 20 mM diacetyl monoxime, and 1.5 mM thiosemicarbazide), followed by vortexing and incubation at 95 °C for 30 min. The absorbance for each reaction was measured at 540 nm and the amount of citrulline produced was determined using a citrulline standard curve. Each assay was done in duplicate and the standard deviation of the duplicate raw data values agreed to within ≤ 20%. The GraFit version 5.0.11 software (Leatherbarrow 2004) was used to fit the data to eq 1.

6.2.7 MALDI-MS Based Activity Assay

The processivity of PRMT1 was determined using a matrix assisted laser
desorption/ionization (MALDI) MS based assay that was previously described (Osborne et al. 2007). Assays were performed in a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 500 μM SAM, and 20 μM AcH4-21 or FOXO1-22. Reactions were then pre-incubated at 37 °C for 10 min followed by the addition of PRMT1 (500 nM final). The reaction was quenched with 3 μL of 50% TFA in ddH2O at the appropriate time point. Spectra were acquired on an Applied Biosystems 4800 Plus MALDI TOF/TOF MS and analyzed using Data Explorer® software. The percent turnover was determined by dividing the intensity of the modified peptide by the sum of the intensities of the unmodified and modified substrates times 100%.

For the PAD4 activity assay, a reaction mixture of 100 mM Tris-HCl pH 7.6, 10 mM CaCl2, 50 mM NaCl, 2 mM dithiothreitol, and 1 mM FOXO1-22 were pre-incubated at 37 °C for 10 min. The reactions were initiated by the addition of PAD4 (0.2 μM final), incubated for 0 min and 60 min at 37 °C, and then quenched by liquid nitrogen. Reactions were desalted using a C18 ZipTip and spectra were acquired using an Applied Biosystems 4800 Plus MALDI TOF/TOF MS. The spectra were analyzed using Data Explorer® software.

6.3 Results and Discussion

6.3.1 Identifying a Minimal Peptide Substrate

FOXO1 is methylated by PRMT1 at R251 and R253 (human numbering) (Yamagata et al. 2008); the original numbers (R248 and R250) are for mouse FOXO1. Because we have previously demonstrated that peptides can be used in place of full proteins for kinetic studies (Osborne et al. 2007), we first sought to identify a minimal peptide substrate for FOXO1. The use of peptides in place of proteins has several
advantages, i.e., the ability to achieve higher concentrations for use in determining kinetic parameters, as well as the ability to easily mimic different post translational modifications. The sequences of the peptides tested against PRMT1 can be found in Table 6.3. Note that AcH4-21 is the minimal peptide substrate previously determined for Histone H4 and was used for kinetic comparison because it is an excellent substrate for PRMT1. All three FOXO1 sequences contain the sites of methylation with variation at the N- and C-termini. The kinetic parameters for these four peptides can be found in Table 6.4.

Table 6.3 Peptide Sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcH4-21</td>
<td>Ac(^{-1})SGRGKGGKGLGKGGAKRHRK(^{21})</td>
</tr>
<tr>
<td>FOXO1-21</td>
<td>Ac(^{-245})KSGKSPRRRASKDNNSFKAK(^{265})</td>
</tr>
<tr>
<td>FOXO1-22</td>
<td>Ac(^{-249})SPRRRASKDNNSFKASKRSAA(^{265})</td>
</tr>
<tr>
<td>FOXO1-30</td>
<td>Ac(^{-245})KSGKSPRRRASKDNNSFKASKRSAAKK(^{265})</td>
</tr>
</tbody>
</table>

Table 6.4 Kinetic parameters of PRMT1 for the FOXO1 peptide substrates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>(K_m) (µM)</th>
<th>Fold</th>
<th>(k_{cat}) (min(^{-1}))</th>
<th>Fold</th>
<th>(k_{cat}/K_m) (M(^{-1})·min(^{-1}))</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcH4-21(^a)</td>
<td>1.1 ± 0.5</td>
<td>-----</td>
<td>4.6 x 10(^{-1}) ± 2 x 10(^{-2})</td>
<td>-----</td>
<td>4.1 x 10(^5)</td>
<td>-----</td>
</tr>
<tr>
<td>FOXO1-21(^a)</td>
<td>110 ± 20</td>
<td>100</td>
<td>4.3 x 10(^{-2}) ± 2 x 10(^{-3})</td>
<td>11</td>
<td>3.8 x 10(^2)</td>
<td>1079</td>
</tr>
<tr>
<td>FOXO1-22(^a)</td>
<td>23 ± 8.5</td>
<td>20</td>
<td>1.46 x 10(^{-1}) ± 9 x 10(^{-3})</td>
<td>3.2</td>
<td>6.4 x 10(^3)</td>
<td>64</td>
</tr>
<tr>
<td>FOXO1-30(^a)</td>
<td>20 ± 13</td>
<td>18</td>
<td>2.0 x 10(^{-1}) ± 2 x 10(^{-2})</td>
<td>2.3</td>
<td>8.6 x 10(^3)</td>
<td>48</td>
</tr>
</tbody>
</table>

\(^a\)[SAM] = 15 µM.

Based on the observed kinetic parameters, the results clearly show that positively charged residues distal to the site of methylation are important for substrate recognition.
For example, there is a ~ 5-fold decrease in $K_m$ between FOXO1-21 and the two longer peptides, which each contain 2 or more additional positively charged residues at their C-termini. This observation is consistent with our hypothesis (see Section 6.1.7) that PRMT1 substrates can be predicted based on the sequence C-terminal to the site of methylation (Table 6.2). Note that while FOXO1-30 has the lowest fold decrease in $k_{cat}/K_m$ (~ 48-fold) relative to AcH4-21, longer peptides tend to be more difficult to synthesize. Additionally, the ~1.3-fold difference in the $k_{cat}/K_m$ values for FOXO1-30 and FOXO1-22 is not significant. Therefore, FOXO1-22 was chosen as the minimal peptide substrate for use in future studies.

### 6.3.2 Processivity of PRMT1 with FOXO1-22

Although previous studies utilized an antibody that was raised towards a FOXO1 peptide that contained asymmetric dimethylarginine at R251 and R253 (Yamagata et al. 2008), the total extent of methylation at these positions (i.e., $\omega$-MMA, ADMA, or a mixture of both) has not been confirmed. It is also uncertain as to whether PRMT1 uses a processive or distributive mechanism with this substrate, i.e., the production of $\omega$-MMA and ADMA before substrate release versus substrate release after $\omega$-MMA formation followed by rebinding of the substrate and SAM for the formation of ADMA. Note that it was previously determined that with the AcH4-21 peptide, PRMT1 uses a partially processive mechanism (Osborne et al. 2007) (Figure 6.4A). The presence of two potentially methylated arginine residues in FOXO1-22, compared to one in AcH4-21, adds an additional level of complexity, because processivity can result from the formation of $\omega$-MMA and ADMA on a single residue or it can also describe whether both arginine residues are sequentially methylated prior to substrate release.
Figure 6.4 Processivity of PRMT1.  (A) With AcH4-21 as a substrate, PRMT1 utilizes a partially processive mechanism in which a second molecule of SAM can rebind to form ADMA prior to release of ω-MMA, but ω-MMA can also be released prior to rebinding of SAM.  (B) The FOXO1-22 peptide contains the addition of up to four methyl groups, however, the majority of methylated peptides contain only 2 methyl groups.

Nevertheless, as an initial effort to better characterize the mechanism of methylation of FOXO1, a MALDI-based mass spectrometry assay was used to assess the formation of the methylation products over time (Figure 6.4). The results show up to four methylation events, which is consistent with two arginine residues being dimethylated. However, methylation appears to be quite slow, with the majority of peptides only containing one or two methyl groups. In order to determine the order of methylation, i.e., whether these two methylation events takes place on the same arginine residue or if ω-MMA is present on both R251 and R253, further analysis using MS/MS will need to be performed.

6.3.3 Effect of Serine Phosphorylation on PRMT1-Dependent Methylation

FOXO1 is phosphorylated by Akt at S256 (human numbering). As stated previously, studies have shown that methylation by PRMT1 prevents phosphorylation but the reverse is not observed (Yamagata et al. 2008). To determine the effect of
phosphorylation on methylation in our peptide model, S256 of FOXO1-22 was substituted with glutamate to mimic phosphoserine (FOXO1-22(S256E)). The kinetic parameters of PRMT1 with this mutant can be seen in Table 6.5. The results show a ~3.1-fold increase in $K_m$ compared to the WT FOXO1-22 peptide and a ~4.9-fold decrease in $k_{cat}/K_m$, thus demonstrating that phosphorylation likely has a small but significant effect on methylation. The discrepancy between our study and Yamagata et al. may lie in the fact that the authors only made a visual observation and thus the conclusion was only relative and not based on quantification. It is also possible that other residues in FOXO1 are critical for methylation, thus demonstrating the need for kinetic analysis with the full length protein to determine if our peptide model is relevant.

Table 6.5 Kinetic parameters of PRMT1 for the FOXO1 peptide substrates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_m$ (µM)</th>
<th>Fold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO1-22</td>
<td>23 ± 8.5</td>
<td>-----</td>
<td>1.46 x 10$^{-1}$ ± 9 x 10$^{-3}$</td>
<td>-----</td>
<td>6.4 x 10$^3$</td>
<td>-----</td>
</tr>
<tr>
<td>(S256E)$^a$</td>
<td>72 ± 13</td>
<td>3.1</td>
<td>9.8 x 10$^{-2}$ ± 4 x 10$^{-3}$</td>
<td>1.5</td>
<td>1.3 x 10$^3$</td>
<td>4.9</td>
</tr>
</tbody>
</table>

$^a$[SAM] = 15 µM.

6.3.4 FOXO1 as a PAD4 Substrate

As mentioned previously, because the PAD family of enzymes also modifies arginine residues, there is the possibility for crosstalk between deimination and phosphorylation. To test whether FOXO1 is a potential substrate for PAD4, kinetic parameters were determined with the FOXO1 peptides. Benzoyl arginine ethyl ester (BAEE), an excellent PAD4 substrate, is used for comparison (Table 6.6). The data show that all three FOXO1 peptides are good substrates for PAD4. Previous studies in our lab
with PAD4 have demonstrated that the enzyme is capable of deiminating multiple arginine residues on a single peptide, thus the slower $k_{\text{cat}}$ values for FOXO1-22 may be due to the lack of two additional arginine residues compared to the other peptides. To determine the number of arginine residues that are converted to citrulline on FOXO1-22 by PAD4, the enzyme was reacted with the peptide for 1 h before being analyzed by MALDI-MS (Figure 6.5). A mass shift of 3 Da indicates that three arginine residues are

![Figure 6.5 Mass spectrometric analysis of the deimination of FOXO1-22 by PAD4. A 3 Da shift is observed between (A) the control reaction minus PAD4 and (B) plus PAD4, thus demonstrating the conversion of three arginine residues to citrulline.]

Table 6.6 Kinetic parameters of PAD4 for the FOXO1 peptide substrates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_m$ (mM)</th>
<th>Fold</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>Fold</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$·min$^{-1}$)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEE</td>
<td>0.4 ± 0.10</td>
<td>------</td>
<td>6.2 ± 3 × 10$^{-1}$</td>
<td>------</td>
<td>1.6 × 10$^{4}$</td>
<td>------</td>
</tr>
<tr>
<td>FOXO1-21</td>
<td>0.2 ± 0.08</td>
<td>0.5</td>
<td>6.8 × 10$^{-1}$ ± 9 × 10$^{-2}$</td>
<td>9.1</td>
<td>3.3 × 10$^{3}$</td>
<td>4.7</td>
</tr>
<tr>
<td>FOXO1-22</td>
<td>0.2 ± 0.03</td>
<td>0.5</td>
<td>4.5 ± 3 × 10$^{-1}$</td>
<td>1.3</td>
<td>2.3 × 10$^{4}$</td>
<td>0.7</td>
</tr>
<tr>
<td>FOXO1-30</td>
<td>0.4 ± 0.07</td>
<td>1</td>
<td>2.8 ± 2 × 10$^{-1}$</td>
<td>2.2</td>
<td>6.3 × 10$^{3}$</td>
<td>2.5</td>
</tr>
</tbody>
</table>
converted to citrulline, as this modification adds 1 Da to the residue mass. Because all three peptides contain at least three arginine residues, it remains uncertain as to whether the slower catalysis observed with FOXO1-21 is due to a smaller number of arginine residues or a difference in substrate recognition elements.

6.3.5 Effect of Serine Phosphorylation on PAD4-Dependent Deimination

Because FOXO1 is a potential substrate for PAD4 based on our peptide model, we next wanted to investigate the effect of phosphorylation on deimination. As with PRMT1, the kinetic parameters were determined for PAD4 with the S256E mutant (Table 6.7). Interestingly, the results show that phosphorylation has a very similar effect on PAD4-dependent deimination as it did with PRMT1-dependent methylation with an ~4.7-fold decrease in $k_{\text{cat}}/K_m$ that can be attributed to an increase in $K_m$. These results suggest that the presence of a negatively charged residue or residues C-terminal to the potentially modified arginine residue(s) has a negative effect on substrate recognition for both PRMT1 and PAD4.

**Table 6.7 Kinetic parameters of PAD4 for the FOXO1 peptide substrates**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_m$ (mM)</th>
<th>Fold</th>
<th>$k_{\text{cat}}$ (min⁻¹)</th>
<th>Fold</th>
<th>$k_{\text{cat}}/K_m$ (M⁻¹·min⁻¹)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO1-22</td>
<td>0.2 ± 0.03</td>
<td>-----</td>
<td>4.5 ± 3 x 10⁻¹</td>
<td>-----</td>
<td>2.3 x 10⁴</td>
<td>1.1</td>
</tr>
<tr>
<td>FOXO1-22 (S256E)</td>
<td>0.87 ± 0.09</td>
<td>4.4</td>
<td>4.3 ± 4 x 10⁻¹</td>
<td>1</td>
<td>4.9 x 10³</td>
<td>4.7</td>
</tr>
</tbody>
</table>

[a][SAM] = 15 μM.

6.4 Conclusions

There are currently several examples of crosstalk between arginine methylation and serine/threonine phosphorylation (*vide supra*). Based on these examples and our
knowledge of PRMT1 catalysis, we have hypothesized that this form of crosstalk is a common mechanism of regulation in cell signaling pathways and that it can likely be predicted based on the sequence surrounding an Akt consensus motif (Table 6.2). Before exploring our theory, we first wanted to better characterize an already known crosstalk model, specifically that of FOXO1, to use as a basis for subsequent studies.

*In vitro* and *in vivo* studies have demonstrated that FOXO1 is methylated at R251 and R253. These sites of methylation have only been confirmed by mutation to lysine residues and the use of an antibody that was raised towards a peptide that contained asymmetric dimethylarginine at these positions (Yamagata et al. 2008). Therefore, confirmation by mass spectrometry is still needed and is currently underway in our lab. To further characterize the mechanism of PRMT1-dependent methylation of FOXO1, initial studies were conducted to determine a minimal peptide substrate (because of the ease of working with peptides in comparison to full length proteins) and the fact that the peptide models have worked well for other PRMT1 substrates (*i.e.*, histone H4). FOXO1-22 was determined to be a sufficient peptide substrate, although kinetic studies still need to be conducted with the full length protein to confirm that this model works for this particular substrate. However, the observed results could aid in defining a possible consensus sequence for PRMT1-dependent methylation.

Because the exact methylation products (*i.e.*, ω-MMA, ADMA, or a mixture of both) at R251 and R253 have also not been confirmed by mass spectrometry, initial studies were done to further characterize methylation of these residues. Although a degree of complication exists because there are two potentially methylated residues as opposed to one, a MALDI-based assay was used to quantify the transfer of methyl groups
to FOXO1-22. The data show that up to four methyl groups are added to the peptide, however, the addition of only one or two methyl groups is predominant. Further studies, such as MS/MS, will be needed to confirm the processivity of PRMT1 towards FOXO1.

FOXO1 is phosphorylated at S256 and this phosphorylation event is inhibited by arginine methylation of R251 and R253, however, phosphorylation did not affect methylation (Yamagata et al. 2008). However, the latter conclusion was only based on relative amounts, therefore, we sought to confirm this observation using our peptide substrate. The S256 residue of the FOXO1-22 peptide was exchanged for a glutamate to mimic phosphoserine. Although small, the observed decrease in $k_{cat}/K_m$ (~ 4.9-fold) is significant, suggesting that phosphorylation can subtly modulate PRMT1 activity. This result will need to be confirmed with the full length protein to determine if other residues surrounding the site of methylation compensate for the addition of a phosphate group.

Since PAD4 also modifies arginine residues within proteins, we examined whether our FOXO-22 peptide was a substrate for PAD4. The results show that all three of the FOXO1 peptides are substrates for the enzyme. The PAD4-FOXO1 reaction was also analyzed by mass spectrometry, which showed that three arginine residues are deiminated on the FOXO1-22 peptide. A similar effect was observed with PAD4 compared to PRMT1 in regards to the FOXO1-22(S256E) peptide, thus suggesting a common link between the consensus sequences of arginine modifying enzymes. Future studies will involve trying to confirm if FOXO1 is actually deiminated in vivo.

Overall, the aforementioned studies are only our initial efforts towards proving our crosstalk hypothesis, as much more work needs to be done. Future efforts will include kinetic studies with the full length protein, to confirm that FOXO1-22 can be
used to study the PRMT1-FOXO1 mechanism, and confirmation of the methylation sites by mass spectrometry. Our PRMT1 inactivator, C21, will be used to examine the crosstalk mechanism between methylation and phosphorylation of FOXO1 \textit{in vivo}. We will then proceed in the same manner with other predicted substrates.
CHAPTER 7

Conclusions & Future Directions

Protein post-translational modifications (PTMs) play a variety of roles in the cell by affecting protein-protein interactions, the cellular location of proteins, and protein stability. Due to their importance in normal cellular function, dysregulation of a variety of PTMs has been linked to a plethora of diseases (e.g., cancer and autoimmune diseases), therefore making them effective drug targets. Our lab is focused on arginine modifying enzymes, more specifically the Protein Arginine Methyltransferases (PRMTs). This family of nine isozymes catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the guanidinium moiety of arginine residues in proteins, generating an ω-monomethylarginine residue (ω-MMA), which can then be further methylated to produce either an asymmetrically dimethylated arginine residue (ADMA) or a symmetrically dimethylated arginine (SDMA) residue. Of the nine family members, we are most interested in PRMT1 because it is thought to be responsible for the majority of asymmetrically dimethylated arginine residues in the cell (Tang et al. 2000 & Pawlak et al. 2000). In addition, a variety of studies have suggested that the enzyme plays an important role in the onset of diseases such as cancer, heart disease, and ALS.

In order to begin to better understand the roles that PRMT1 plays in healthy and diseased cells, we first investigated the catalytic mechanism of the enzyme. Using site-directed mutagenesis, we confirmed the importance of several active site residues to catalysis (i.e., E153 and H293) and substrate/cofactor binding (i.e., M155 & H293). We
also determined that H293 likely does not act as a general base in the mechanism. Finally, our results suggest that prior deprotonation of the guanidinium is not required and the reaction is simply catalyzed by bringing the cofactor and substrate into close proximity.

We next set out to probe the means by which PRMT1 activity is regulated. Although several of the PRMT family members have been found to be regulated by PTMs, the regulation of PRMT1 by this method has yet to be revealed. According to curated mass spectrometry data from PhosphoSitePlus® (www.phosphosite.org), PRMT1 is potentially phosphorylated, ubiquitylated, and acetylated in the cell (Hornbeck et al. 2012). As a starting point, we investigated two possibly phosphorylated residues, i.e., S102 and Y291. Using site-directed mutagenesis and the incorporation of pCMF, a stable phosphotyrosine mimic, we determined the effect that phosphorylation of these individual residues would have on activity. The S102 residue, which is conserved amongst Type I PRMTs, is important for CARM1 activity and phosphorylation of this residue prevents SAM binding and abolishes activity. The results show, however, that phosphorylation of S102 does not affect PRMT1 activity and thus modification of this residue is likely not a mechanism for regulating this enzyme. The Y291 residue, which was chosen based on PhosphoSitePlus® (www.phosphosite.org) data, appears to play a role in substrate binding, as demonstrated by increases in the $K_m$ values for the Y291A, Y291E, and Y291pCMF mutants. Although we hypothesized that phosphorylation of this residue would greatly affect overall activity, especially SAM binding, the results suggest that the interaction between H293 and D51 is enough to hold the N-terminal $\alpha$-helix in place for proper SAM binding. It is also possible that pCMF does not fully
mimic phosphotyrosine because it is lacking a negative charge and a greater effect on SAM binding would be observed in vivo. However, based on studies showing that the N-terminal splice variants of PRMT1 have different substrate specificities, we hypothesize that phosphorylation of this residue may destabilize the N-terminal tail and alter the substrate specificity. Future studies will involve, not only further investigation into the effect of phosphorylation of Y291, but also the effect of other potentially phosphorylated PRMT1 residues, and a search for the responsible kinases.

PTMs are not the only means of enzyme regulation, as protein-protein interactions have also been found to affect the activity of the PRMTs. PRMT1 has two known interacting proteins, CAF1 and BTG1, which appear to inhibit and/or enhance activity in a substrate specific manner (Lin et al. 1996, Berthet et al. 2002, Robin-Lespinasse et al. 2007). To characterize the effects of these two proteins from a mechanistic point of view, we performed assays in vitro with increasing concentrations of CAF1 or BTG1. The results suggest that CAF1 binds to histone H4 and inhibits methylation, but it can also bind PRMT1 and target it to the histone. On the other hand, BTG1 appears to bind both PRMT1 and histone H4, but does not inhibit activity. Future studies will involve examining the effects of these two proteins in regards to other histones, as well as, immunoprecipitation experiments to confirm that they can interact with PRMT substrates.

PRMT1 appears to be involved in a variety of diseases, and thus one of our main goals is to develop potent and selective inhibitors and inactivators for the enzyme. We have previously described C21, the most potent PRMT1 inhibitor to date (Obianyo et al. 2010); however, it was not known which residue was covalently modified. MS/MS was
used to identify an active site cysteine, C101, as the site of modification. Although this residue is not important for catalysis, as demonstrated by the fact that the kinetic parameters are similar to the WT enzyme, other studies have shown that it is a hyper-reactive cysteine (Weerapana et al. 2010). Interestingly, only PRMT1 and PRMT8 have an active site cysteine residue at this position, as PRMT3 and CARM1 each have a cysteine residue that is buried away from the active site. In an attempt to target C101 and potentially develop a new PRMT1 selective inhibitor, we coupled a chloroacetamidine warhead to the adenosine portion of SAM to create Cl-acetamidino-N-ethyl-aminoadenosine. Although it is not nearly as potent as C21, it is irreversible, and comparison of the IC$_{50}$ for the compound with N-ethyl-aminoadenosine and adenosine show that the warhead is essential for inhibition. As this is only a first generation inhibitor, future studies will involve designing and synthesizing SAM analogues based on SAH with a warhead attached. In addition, PRMT5 has an active site cysteine residue that is in the SAM binding pocket but on the opposite side of C101. We hypothesize that we will be able to create selective inhibitors for both of these enzymes by fine tuning the position of a warhead on SAH.

Finally, there are several examples that demonstrate crosstalk between arginine methylation and phosphorylation within kinase consensus sequences, more specifically between PRMT1 and Akt. In the case of FOXO1, studies have shown that methylation of R251 and R253 prevents the phosphorylation of S256 by Akt, however, prior phosphorylation does not affect methylation (Yamagata et al. 2008). Based on this example, and several others, and examination of the sequences of Akt substrates, we hypothesize that we can predict crosstalk between these two modifications. We have
previously shown that positively charged residues C-terminal and distal to the site of methylation are important for substrate recognition (Osborne et al. 2007). Therefore, the presence of positively charged residues C-terminal to arginine residues within kinase consensus sequences are likely an indicator that the substrate can be methylated by PRMT1 and that this methylation inhibits phosphorylation. Before testing predicted substrates, we first set out to characterize crosstalk in regards to FOXO1. Analysis of the kinetic parameters of PRMT1 with peptides based on the methylated sequence of FOXO1 confirmed our suspicion that positively charged residues are critical. The results also showed that prior phosphorylation does have a small effect on substrate binding. In addition, the FOXO1 peptides were found to be good substrates for PAD4, an enzyme which converts arginine to citrulline in proteins. Future studies will be focused on confirming the sites of methylation with the full length FOXO1 protein, examining crosstalk between methylation and phosphorylation of FOXO1 in vivo with C21, our PRMT1 inhibitor, attempting to identify deiminated FOXO1 in vivo. We will also use these same methods to attempt to identify crosstalk within our predicted PRMT1 substrates.

In conclusion, the results presented in the previous chapters have given us further insight into the catalytic mechanism of PRMT1-dependent methylation and the regulation of enzyme activity by phosphorylation and protein-protein interactions. They have also laid the groundwork for the development of more potent and selective inhibitors for PRMT1 and PRMT5, as well as, the prediction of crosstalk between arginine methylation and phosphorylation.
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