POP2: A POTENTIAL REGULATOR OF HMT1-CATALYZED ARGinine METHylation IN YEaST

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE in

Biochemistry

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2014
ABSTRACT

Pop2: A Potential Regulator of Hmt1-Catalyzed Arginine Methylation in Yeast

by

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Utah State University, 2014

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Protein arginine methylation is an important post-translational modification that is vital in regulating various cellular processes such as gene transcription, cell signaling, and RNA processing. Protein arginine methyltransferases (PRMTs) are responsible for performing this important modification. PRMT1 (protein arginine methyltransferase 1) and Hmt1 (hnRNP methyltransferase 1) are the predominant PRMTs in humans and yeast, respectively. Despite growing momentum in this field, relatively little is understood about PRMT regulation. Further work discovering how PRMTs are regulated will greatly advance our understanding of diseases where PRMTs have been implicated, such as heart disease, viral pathogenesis, and cancer.

It has been discovered that a human protein called hCaf1 (human Ccr4-associated factor 1) is a regulator of PRMT1 with respect to certain substrates, and also colocalizes with PRMT1. We present data that suggest the yeast homolog of hCaf1, Pop2, may also perform a similar function on Hmt1. We provide data on the expression and purification of a truncation of Pop2 from S. cerevisiae, including the temperature
sensitivity of one construct of Pop2 and its susceptibility to precipitation. We also demonstrated concentration-dependent inhibition of Hmt1-catalyzed methylation of histone H4 by Pop2 in vitro. Yeast cell lysates also showed altered patterns of methylation in the presence and absence of Pop2 in vivo. In an effort to understand the mechanism employed by Pop2 to accomplish this regulatory function, pull-downs were performed suggesting that Pop2 directly interacts with histone H4, a substrate of Hmt1. Mutagenic studies with Pop2 suggested a region that may be responsible for this interaction. Given these data, we hypothesized that Pop2 is able to inhibit the methylation of histone H4 via a substrate-sequestering mechanism. Further experimentation will determine the precise interaction surfaces of Pop2 and substrate, and continue to define the details of methylation inhibition by Pop2, including the scope of its influence in the cell.

(73 pages)
PUBLIC ABSTRACT

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Celeste Excell

Enzymes are ubiquitous within our cells, and perform a variety of essential functions as they catalyze important biochemical reactions that allow us to develop, grow, adapt, and live. Each enzyme has a unique function, but these functions often need to be regulated. PRMTs (protein arginine methyltransferases) are one family of enzymes that perform a specific type of modification (arginine methylation) on many proteins (substrates), playing a role in numerous biological processes. PRMTs are so widespread and vital to the cell that dysregulation of these enzymes has profound detrimental effects, implicating PRMTs in several human diseases such as cardiovascular disease and cancer. This emphasizes the urgency in understanding how PRMTs are regulated. A human protein, hCaf1, was discovered to regulate the activity of PRMT1, the predominant PRMT in humans, with respect to certain substrates. We investigated the yeast homolog of hCaf1, Pop2, and hypothesized that it would perform a similar function in yeast. This thesis presents both in vitro and in vivo data that confirm the regulatory function of Pop2 on Hmt1, the yeast homolog of PRMT1. We delved into the mechanism that Pop2 employs to accomplish this regulation. We demonstrated that Pop2 unexpectedly interacts with a substrate of Hmt1, suggesting a substrate-sequestering mechanism. This and future research will contribute to the foundational knowledge of PRMT regulation, allowing the development of specific PRMT regulators to treat and prevent human diseases where PRMTs have been implicated.
ACKNOWLEDGMENTS

I would like to take this opportunity to thank Dr. Joanie Hevel, my advisor and mentor, for all her help, instruction, and encouragement throughout my graduate education. I would also like to thank my committee members, Dr. Sean Johnson and Dr. Liaohai (Leo) Chen, for all of their assistance and guidance throughout the entire process. I would also like to thank the professors and teachers of the graduate courses for all they taught me during my time here at Utah State University. I am also very grateful to my fellow lab members and colleagues for all of their help and friendship. I would especially like to express gratitude for my wonderful husband, Bryan, for his continued loving patience, support, and encouragement.

Celeste Excell
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CHAPTER 1

INTRODUCTION

Overview

Communication is a vital component of our world, from international news down to intracellular signals. The exchange of information on any level requires some kind of language, a means for transmitting this necessary information. Just as nations have written and spoken languages, and computers have binary and algorithms, cells have languages all their own. From the smallest bacteria to a complex human being, cellular communication is a requirement for life. Discovering how cells and cellular components talk to each other has been an ongoing endeavor for decades.

The scientific community has gained an extensive understanding of many aspects of cellular communication and signaling, including the expansion of the proteome through post translational modifications (PTMs). PTMs are covalent modifications of proteins that alter their function. Examples of these modifications include phosphorylation, acetylation, methylation, ubiquitination, lipidation, glycosylation, and many others. PTMs play a valuable role in regulating transcription and signal transduction, altering protein interactions and localization, influencing enzymatic activity, protein stability and folding, and many other cellular processes. Considering the pervasiveness and importance of PTMs to so many aspects of cellular physiology, it is crucial to gain as much knowledge of them as possible in order to better understand not only how cells function, but also how cells malfunction. This information is then utilized in developing prevention strategies and pharmaceuticals to treat those malfunctions that cause human disease and illness.
SAM-Dependent Methylation

One of the PTMs mentioned above is methylation, and it is the fourth most common type of PTM based on putative methylation sites. Protein methylation can occur on the C- or N-termini of polypeptides as well as on amino acid side chains, typically arginine and lysine. These methylations are performed by a class of enzymes called methyltransferases (MTases), which transfer a methyl group (-CH₃) from a methyl group donor to the substrate. MTases that rely on S-adenosyl-methionine (SAM, or AdoMet) as the methyl group donor are referred to as SAM-dependent, or AdoMet-dependent MTases.

SAM is a widespread substrate, perhaps second only to ATP, and participates in a variety of reactions including decarboxylation and hydroxylation reactions, adenylyl transfer, and even radical mechanism reactions. SAM also serves as a precursor to numerous compounds. However, its predominant function is methyl group donation to proteins, DNA, RNA, and numerous other substrates, such as those involved in arsenic metabolism, and in the synthesis of plant sterols, methyl halides, methanethiol, and other compounds. With a wide variety of substrate targets, versatile applications, and favorable energetics (-17 kcal/mol), SAM makes an ideal methyl donor, although there are others that are less common such as N⁵-methyltetrahydrofolate.

Most SAM-dependent MTases contain a conserved SAM-binding domain, a Rossman fold, or “AdoMet-dependent MTase fold,” despite a lack of sequence identity among them. These binding domains place SAM in an in-line position to their respective bound substrates so that the methyl group of SAM can be transferred to the substrate via an S_N₂-type reaction mechanism, resulting in a stereochemical inversion of the methyl group. The positive charge of the sulfonium group of SAM assists in making the methyl group more susceptible to nucleophilic attack by the substrate. Once
the methyl group is donated, the enzyme releases S-adenosyl-homocysteine, (SAH, or AdoHcy) and the newly modified protein as products of the reaction (Figure 1-1).

![Figure 1-1. Structures of SAM and SAH](image)

**Figure 1-1. Structures of SAM and SAH** (A) S-adenosyl-L-methionine, abbreviated as SAM, or AdoMet, and (B) S-adenosyl-L-homocysteine, abbreviated as SAH, or AdoHcy.

**Arginine Methylation**

As mentioned above, arginine is one of the most common amino acid residues to be methylated. Arginine has several characteristics that make it unique among the amino acids, such as the ability to participate in five different hydrogen bonds. Arginine’s interactions with DNA, RNA, and other proteins are made possible by its ability to participate in ionic salt bridges, cation-π interactions, and Van der Waals interactions.

Arginine methylation is also a rather unique PTM, and two facts emphasize the evolutionary significance of methylated arginines: they are only found in eukaryotes, and each methylation event is very energetically expensive, requiring the utilization of 12 ATP. At such a high energetic cost, the cell must greatly value and rely on the PTM of arginine methylation.
Although a methyl group may seem small, its effects can have a significant impact on protein-DNA, protein-RNA, and protein-protein interactions. A methyl group does this by eliminating hydrogen bond potential, adding bulkiness and steric hindrance, and increasing hydrophobic interactions with other residues such as tryptophan.\textsuperscript{2} In fact, the interaction between dimethylated arginine and tryptophan is twice as strong as the same interaction with an unmethylated arginine.\textsuperscript{19} The altered interactions are important for a variety of functions: signal transduction, RNA splicing and processing, nuclear export, DNA repair, chromatin organization, regulation of transcription, and many others.\textsuperscript{2,3,16,17,20–25}

Another distinctive feature of arginine is that it has multiple nitrogen atoms that are capable of being methylated. This gives rise to the four different forms of methylated arginine, the most prevalent of which is asymmetric dimethyl arginine (ADMA) (Figure 1-2).\textsuperscript{2,16} The other forms include symmetric dimethyl arginine (SDMA), and monomethyl arginine (MMA). These modifications all occur on the terminal guanidino nitrogens of arginine. However, the fourth form has the δ-nitrogen of the guanidino group monomethylated, termed δ-MMA (not shown in the figure), and has only been reported in yeast.\textsuperscript{2,26}

**Protein Arginine Methyltransferases (PRMTs)**

The family of enzymes that is responsible for arginine methylation is the protein arginine methyltransferase (PRMT) family. In mammals, there are 11 different PRMTs, and they are categorized by the type of modified arginine they produce (Figure 1-2). Type I PRMTs catalyze the reaction that produces MMA and ADMA, and these include PRMTs 1, 2, 3, 4/CARM1, 6, and 8.\textsuperscript{26,27} Type II PRMTs produce MMA and SDMA, and these include PRMT5, and possibly 9.\textsuperscript{16,26} Type III PRMTs are responsible for the
production of solely MMA, and this category includes PRMT7. PRMT10 and 11 have been identified by their homology with PRMT7 and 9, but their specific PRMT activity is yet to be determined by experimental evidence.

In mammals, the predominant PRMT is PRMT1 which is responsible for about 85% of the arginine methylation that occurs. PRMT1 has seven isoforms, each of which is altered in the N-terminal sequence. These isoforms are tissue-specific, and vary in their substrate specificity and enzymatic activity. The prevalent balance and expression of these isoforms has been implicated in several different human diseases, including cancer.

**Structural Insights into PRMTs**

Crystal structures of various PRMTs have been solved, which greatly contribute to our current understanding of PRMTs and how they function. Throughout all of these structures, the catalytic core of the PRMT enzymes is conserved. This catalytic

![Figure 1-2. PRMT Methylation Products.](image)

Protein arginine substrates are modified to form MMA, ADMA, and SDMA by type III, type I, and type II PRMTs, respectively.
core is comprised of a SAM-binding domain, a β-barrel domain, and a dimerization arm, with the active site nestled in a deep pocket between the SAM-binding and β-barrel domains. The active site also contains a strictly conserved hairpin called the “double E loop,” where two catalytic glutamates reside. These studies also provide evidence that homodimerization of PRMTs is required for SAM binding and catalytic activity.29–34

The structure of PRMT1 is of particular interest as it is the predominant PRMT in mammals, and structural and enzymatic studies of its wild type and mutant forms have unveiled many valuable pieces of information about its mechanistic and functional characteristics.27,32,35,36 In order to gain a better understanding of this important workhorse of the PRMT family, the yeast homolog of PRMT1 from Saccharomyces cerevisiae (Hmt1/Rmt1) can also provide valuable insights into this enzyme in the context of a more simple eukaryotic system, and will be the focus of this thesis.

**Protein Arginine Methyltransferases in Yeast**

The predominant PRMT in yeast (S. cerevisiae) is Hmt1/Rmt1. As the yeast homolog of PRMT1, Hmt1 is also a type I PRMT, catalyzing the formation of MMA and ADMA. Hmt1 is responsible for 89% of the ADMA and 66% of the MMA formed in yeast in vivo.2

The crystal structure of Hmt1 has been solved, and shows many similarities to PRMT1.33 Hmt1 forms a homodimer that is essential for activity, mediated by its dimerization arm, or “antenna.” This antenna is a small, two helix bundle, and when mutated to a series of alanines, Hmt1 loses its ability to dimerize, as well as its catalytic activity.33 Gel filtration experiments show that both PRMT1 and Hmt1 form a hexamer in solution, and both crystallize as a trimer of dimers, although the functional role of the hexamer is unclear.22,33
Other PRMTs in yeast include Rmt2, Hsl7 (the yeast homolog of PRMT5), and Sfm1. Rmt2 is a unique PRMT in that it catalyzes the addition of a methyl group to the δ-nitrogen of arginine instead of one of the ω-nitrogens, making it a type IV PRMT. Although this type of methylation is present in other lower fungal species, it is not conserved in higher eukaryotes. One of Rmt2’s known substrates is a ribosomal protein L12, and Rmt2 also associates with specific nucleoporins, but details of its roles in the cell remain unclear.

Hsl7, like PRMT5, is a type II PRMT and catalyzes the formation of MMA and SDMA. Although in vivo substrates of this enzyme are unclear, it is known to be important in cell cycle regulation, and is also important for cell survival under hyperosmotic stress.

Sfm1 is the fourth and last of the currently known PRMTs in yeast. It has been tentatively classified as a type III PRMT since it has only been shown to monomethylate a small ribosomal protein, Rps3. The specific functional role of this modification has yet to be elucidated. In addition to the four PRMTs currently documented in yeast, there is still PRMT activity that is unaccounted for, suggesting that there are other PRMTs that have yet to be characterized in this species.

Despite the prevalence of other PRMTs in yeast, our focus is on Hmt1, the yeast homolog of PRMT1. This project is mainly concerned with discovering how Hmt1 is regulated in hopes that our findings can be used in studying PRMT1 and uncovering potential avenues for treating and preventing diseases related to PRMT dysregulation. The studies in this thesis involve a yeast protein, Pop2, and its role as a potential regulator of Hmt1.
Substrates and Roles of Protein Arginine Methylation

PRMTs typically methylate arginines located in glycine-arginine-rich (GAR) motifs, usually in the form of RGG sequences, although there are exceptions to this rule. For example, PRMT1 performs methylations in RXR sequences, and in some RGX and RXG sequences.\(^2,16,26\)

RNA binding proteins (RBPs) represent a major substrate of PRMTs as a result of most of them harboring GAR motifs, and many of them have been identified as being arginine methylated.\(^22\) Two of these RBPs in yeast, Npl3 and Nab2, shuttle RNA from the nucleus to the cytoplasm, and this function has been shown to be methylation dependent.\(^2,40\) In mammals, the methylation status of other RBPs, such as Sam68 and RNA helicase A which are methylated by PRMT1, determines their subcellular localization and contributes to their function and regulation.\(^20,41\)

As mentioned earlier, methylated arginines play important roles in a variety of cellular functions in addition to RNA processing. Ribosomal proteins have also been identified as substrates of arginine methylation, and this PTM is evolutionarily conserved.\(^2\) As a modification that is growth-condition dependent in yeast, and cell cycle-dependent in HeLa cells, it appears that ribosomal arginine methylation plays an important role in translational regulation.\(^42,43\)

Signal transduction is another area where arginine methylation has been shown to play an important role by strengthening or blocking certain interactions.\(^22\) A few examples of signaling pathways that utilize arginine methylation include T-cell, interferon, cytokine, and nerve growth factor signaling.\(^22,44\) Recently, a study has shown that the Smad signaling cascade also involves PRMT1 activity in order to transmit the signal from the membrane bound receptors that bind BMPs (bone morphogenetic proteins) into the cell, which influences levels of gene transcription.\(^45\)
Some substrates of PRMTs are involved in DNA damage checkpoint control and repair. MRE11 is a substrate of PRMT1 within its GAR domain, and the presence of the ADMA modification regulates the exonuclease activity of MRE11. The p53 protein is methylated by PRMT5 in response to DNA damage, which influences the regulation of subsequent cell cycle arrest. These results among others illustrate the valuable role of arginine methylation in regulating the cell’s response to DNA damage and repair.

One of the more well-studied areas of arginine methylation is the methylation of histones, and the subsequent effects this modification has on transcriptional regulation. To date, there are five PRMTs in mammalian systems that are known to methylate histones. Histone sites H3R17 and H3R26 are asymmetrically dimethylated by PRMT4, and PRMT6 also asymmetrically dimethylates H3R2. PRMTs 1, 6, and 8 modify H4R3 and H2AR3, and these modifications are associated with actively transcribed promoters. PRMT5 symmetrically dimethylates H4R3, H2AR3, and H3R8. These modifications by PRMT5 are linked to transcriptional repression and gene silencing. Since both PRMT1 and PRMT5 methylate H4R3 in different ways (ADMA and SDMA, respectively), it is reasonable that the two enzymes produce antagonistic effects; one (ADMA) turns transcription of a gene on while the other (SDMA) turns it off. Conversely in yeast, Hmt1-catalyzed asymmetric dimethylation of H4R3 is shown to repress transcription and is involved in silent chromatin formation. Methylation of histone H4R3 by Hmt1 is a primary focus of this project, and our main endeavor has been discovering how methylation of this particular substrate can be regulated by Pop2, another yeast protein.

Another interesting aspect of histone arginine methylation is its interactions and interplay with other histone modifications, such as acetylation and lysine methylation. Hmt1 has been shown to preferentially bind and methylate acetylated histones. This
theme of acetylation and methylation cooperatively interacting seems to be conserved in mammals as well. When PRMT1 was knocked down in an erythroid cell line, there was a loss of acetylation on both histones H3 and H4, as well as an increase in lysine methylation. Lysine methylation is a well-characterized PTM of histones, and is hypothesized to crosstalk with arginine methylation sites in the form of “arginine/lysine-methyl/methyl switches.” In these switches, a methylated arginine or lysine influences the subsequent modification of another adjacent arginine or lysine. Although some details remain elusive of how arginine methylation of histones functions in the context of epigenetics, it is clear that it plays an important role in regulating transcription as part of the histone code.

PRMTs can also influence transcriptional regulation through methylation of non-histone proteins. In mammals, co-activators of transcription can be methylated, which leads to the modification of histones and subsequently altered transcription levels. A more direct method documented in mammals is methylation of transcription factors that can activate gene transcription. In yeast, Hmt1 can affect transcription elongation and termination by methylating anti-terminator and terminator proteins such as Npl3 and Hrp1, respectively.

These findings among many others make it clear that from yeast to humans, PRMTs carry critical responsibility in regulating transcription for proper cell functioning. Discovering how PRMTs are regulated in the context of transcriptional regulation and other cellular processes is critical for future therapeutic and pharmaceutical applications. We are particularly interested in the methylation of histone H4 in yeast, as it is one of the more prominent substrates of Hmt1. This project aims to discover how Hmt1-catalyzed methylation of histone H4 is regulated, as this particular PTM can have very far-reaching and widespread consequences in terms of transcriptional regulation.
PRMT Regulation

Compared to the rest of the collective knowledge of PRMTs, less is known about their regulation. Arginine methylation is considered to be a mostly irreversible PTM, since no true demethylases have been identified thus far. Considering the somewhat permanent nature of arginine methylation in combination with the high energetic cost of each methylation event (12 ATP), it must be extremely important to the cell to tightly regulate this PTM.\(^{17,18}\) Currently known methods of regulation include PRMT-binding proteins and small molecules, oligimerization, deimination and demethylimination, adjacent PTMs of substrates, and direct modification of PRMTs with PTMs.\(^{2,3,24,38,57-60}\) These methods of regulation can activate, enhance, and inhibit enzymatic activity, or alter substrate specificity.\(^2\) This project explores a novel function of a yeast protein, Pop2, as a regulator of Hmt1-catalyzed arginine methylation. We are interested in the ability of Pop2 to modify the activity of Hmt1 toward certain substrates such as histone H4. Before that is discussed, however, a brief summary of other regulatory methods of PRMTs is provided below.

Protein-protein interactions utilized in PRMT regulation are known to occur with multiple PRMTs, both mammalian and yeast. The enzymatic activity of PRMT1 is enhanced when interacting with PRMT2, affecting its \(K_m\) and \(V_{max}\) values.\(^ {61}\) Activity of PRMT1 is also increased when bound to BTG1 or BTG2 proteins with respect to certain substrates.\(^ {62}\) Human Ccr4-associated factor 1 (hCaf1), a BTG1-binding protein, has been shown to co-localize with PRMT1 \textit{in vivo}, and regulate the methylation of histone H4 and Sam68 substrates.\(^ {57}\) hCaf1 is the human homolog of the yeast Pop2 protein, and its relationship with PRMT1 will be discussed in more detail later on.

Another example of protein interaction regulation is PRMT4/CARM1 and its ability to methylate nucleosomal histone H3 when it is part of the NUMAC complex.
(nucleosomal methylation activation complex), as opposed to preferentially methylating free histone H3 when not associated with NUMAC. One domain of the DAL-1/4.1B tumor suppressor protein associates with PRMT3, and inhibits methylation of one of its substrates. The substrate specificity of PRMT5 is modified by cooperator of PRMT5 (COPR5). When COPR5 bound to PRMT5, histone H4 (Arg 3) was methylated preferentially over histone H3 (Arg 8), both of which are substrates of PRMT5.

Although these are just a few examples, protein-protein interactions are a widespread and well-documented method of regulating PRMTs.

Oligimerization of PRMTs is another method of regulating arginine methylation. Homodimerization and multimerization have been shown to be essential for some of the PRMTs including Hmt1, PRMTs 1, 4, and 5. As a result, anything that disrupts these oligimerization interactions contributes to reduced or no enzymatic activity of the PRMT. A cell could use post translational modifications, oxidation/reduction, or other means to regulate PRMT oligimerization.

Demethylation could potentially be another method of regulating arginine methylation, though no true arginine demethylases have been identified as of yet. The discovery of lysine demethylases and their importance in regulating histone lysine methylation were critical advancements in our understanding of the reversibility of methylation. The existence of lysine demethylases and therefore lysine demethylation implies that arginine methylation reversal may be a viable means of PRMT regulation despite the lack of a known arginine demethylase enzyme. Several years ago, a study reported that a Jumonji-domain containing protein, JMJD6, displayed histone demethylase activity. However, other laboratories were unable to replicate these results, and characterized JMJD6 as a lysine hydroxylase enzyme. Further work is needed to continue searching for a true arginine demethylase in humans. As of now, no
arginine demethylase has been reported in yeast.\textsuperscript{2} Two of the currently known lysine
demethylases, Jdh1 and Jdh2, are conserved in S. cerevisiae, suggesting there could be
an analogous undiscovered arginine demethylase in yeast.\textsuperscript{2,74,75}

Somewhat similar to demethylation, deimination is a PTM that prevents an
arginine methyl modification of a substrate by converting an arginine residue to citrulline.
The enzymes that catalyze this reaction are peptide arginine deiminases (PADs), and
the core histones are one of the major groups of proteins that are deiminated.\textsuperscript{18,23} A
substrate of PRMT4, p300, has also been shown to be deiminated by PAD4.\textsuperscript{24} Experimental evidence is contradictory whether PADs can perform demethylimination
reactions (converting MMA, SDMA, or ADMA into citrulline).\textsuperscript{76–78} At best, PADs may do
so very inefficiently \textit{in vitro}.\textsuperscript{58} In addition, the deimination reaction could be an
irreversible modification, as there has not yet been an enzyme identified that can convert
citrulline back into an arginine residue.\textsuperscript{58} Although PADs are not true “demethylases,”
they are prevalent in mammalian cells and may play a vital role in PRMT regulation in
mammals. However, there has not yet been any evidence of demethylimination or
deimination in yeast.\textsuperscript{2}

PTMs directly on PRMTs are another method of regulating their enzymatic
activity. Some PRMTs, such as PRMT6 and 8, are automethylated, but the physiological
function of this PTM is still unclear.\textsuperscript{3,79} PRMT4 has been shown to be phosphorylated,
which reduces its enzymatic activity by disrupting its ability to dimerize.\textsuperscript{67} Hsl7 has also
been shown to be phosphorylated, although this modification in yeast did not seem to
affect its enzymatic activity.\textsuperscript{80} Recently, Hmt1 was shown to be phosphorylated on serine
9, and this was hypothesized to be an essential PTM for Hmt1 oligimerization and
activity.\textsuperscript{59} The Hmt1 S9E mutant (a phosphorylation mimic) was shown to be
constitutively active, even under starvation and rapamycin-treated conditions, whereas wild type Hmt1 was not active under these conditions.\textsuperscript{59}

PTMs on substrates, either adjacent to or directly on the arginine in question, can also regulate PRMTs. One example of adjacent PTMs was mentioned earlier with arginine/lysine methyl/methyl switches.\textsuperscript{23,55} A recent study with PRMT1 knockdown suggested that different PRMTs may regulate each other by using one form of methylated arginine to preclude the formation of another.\textsuperscript{81} When PRMT1 (a type I PRMT) was knocked down, this resulted not only in reduction of ADMA, but also in an increase of SDMA and MMA (catalyzed by type II and III PRMTs, respectively). This effect was referred to as “substrate scavenging,” and suggests that the ADMA modification catalyzed by PRMT1 may be a form of regulation by preventing other PRMTs from forming MMA or SDMA on those same substrates.\textsuperscript{81}

Perhaps the most drug-target-oriented method of PRMT regulation is that done by small molecules. Specific activation or inhibition of PRMTs could be of great pharmaceutical value in treating a variety of diseases that show dysregulated PRMT activity.\textsuperscript{38} Studies have found small molecules that enhance the enzymatic activity of PRMT4 toward both histone and non-histone substrates.\textsuperscript{60} However, specificity of PRMT modulators as well as other challenges make this endeavor very difficult.\textsuperscript{38} One of the studies utilizing small molecules has shown inhibition of PRMTs specific enough to not affect lysine methyltransferases, although the mechanism is unknown.\textsuperscript{60} Others have modeled potential inhibitors that employ a SAM/SAH analog, substrate analog, or both, which could bind in the active site of specific PRMTs and selectively inhibit their activity.\textsuperscript{38} Further experimentation will be needed for drug-inducible regulation that is either PRMT-specific or substrate-specific to become possible. This is an important aspect of PRMT regulation as inhibition of all or multiple PRMTs could result in serious
and harmful side-effects. Considering how widespread PRMTs are, specificity of regulation is critical.

There are many ways that the cell is able to monitor and regulate the activity of PRMTs, both discovered and undiscovered. Suffice it to say that despite the above described regulatory mechanisms that are currently understood, there is still much ground to cover in fully comprehending this area of PTMs. This project seeks to advance our understanding of PRMT regulation through experiments with Hmt1 and Pop2, a potential new regulator of arginine methylation in yeast.

**Clinical Significance**

PRMTs have been implicated in a wide range of human diseases, including cancer, cardiovascular disease, spinal muscular atrophy, and viral pathogenesis.\(^3\,^22\) Given that arginine methylation is a relatively young field compared to some PTMs, our current understanding of the function of PRMTs in human disease is relatively limited. However, this field is quickly expanding as we begin to realize the pervasive influence of PRMTs in the development and progression of cellular malfunction and disease.

Several PRMTs have been implicated or reported as being dysregulated in many types of cancers. Some of these include mantle cell lymphoma, lymphoid, bladder, and lung cancers, as well as hormone-dependent cancers such as prostate and breast cancers.\(^28\,\!^50\,\!^82\,\!^84\) PRMTs 1 and 4 were shown to be overexpressed in many breast cancer cell lines, in addition to overall increased ADMA levels.\(^28\) PRMTs 1 and 6 were upregulated in a variety of cancers.\(^84\) PRMT4 was also overexpressed in human prostate carcinomas, and contributed to androgen independence.\(^28\,\!^82\) PRMT4’s role in prostate cancer progression marks it as a prime potential drug target in treating these types of cancer.\(^83\)
A tumor suppressor protein, p53, is known to be regulated by two PRMTs: PRMT5 methylates p53, determining whether the cell repairs damaged DNA or undergoes apoptosis; and PRMT6 negatively regulates the transcriptional levels of p53.\textsuperscript{21,85} Studies show that knockdown of these and other PRMTs, such as PRMT5, reduces the proliferation of cancer cells.\textsuperscript{50} This suggests that inhibiting PRMTs could be a potentially powerful drug-target by altering epigenetic modifications of aberrant PRMTs and increasing cancer cells’ sensitivity to chemotherapeutics.\textsuperscript{38,50}

Cardiovascular diseases (CVD) are another area of clinical significance related to PRMTs because of CVDs strong link established with ADMA. Free ADMA is generated from the proteolysis of type I PRMT substrates, and functions as an endogenous inhibitor of nitric oxide synthase (NOS), an enzyme located in blood vessel endothelium responsible for generating nitric oxide (NO) from L-arginine. NO is a vasoactive mediator that contributes to vasodilation and other anti-atherosclerotic functions.\textsuperscript{86} When plasma ADMA levels rise and the ratio of L-arginine to ADMA falls, cardiovascular diseases such as hypertension, stroke, congestive heart failure, coronary artery disease, and diabetes are more likely to result.\textsuperscript{86}

An enzyme that helps to alleviate the inhibitory effects of ADMA is dimethylarginine dimethylaminehydrolase 1 (DDAH1), which is responsible for degrading free ADMA.\textsuperscript{86–88} Inhibition or diminished expression of this enzyme can result in tipping the balance toward more ADMA. This imbalance has been associated with dysfunctional endothelium in the above mentioned disease states, and is hypothesized to be the cause of ADMA-mediated NOS inhibition.\textsuperscript{87} Indeed, DDAH1 knockout mice exhibited accumulation of plasma ADMA and MMA, a decrease in the L-arginine to ADMA ratio, and about 20 mmHg increase in blood pressure compared to wild type mice.\textsuperscript{86} Additionally, other studies have administered ADMA to guinea pigs and rats, which also
resulted in increased plasma ADMA levels coupled with increased blood pressure.\textsuperscript{89} Similar studies have been conducted in humans, resulting in overall diminished NO levels and impaired endothelial function.\textsuperscript{89}

Interestingly, increased ADMA levels have been shown to alter gene expression in endothelial cells.\textsuperscript{90} When human coronary artery endothelial cells were treated with ADMA, genetic expression of bone morphogenetic proteins, ribosomal proteins, PRMT3, and other genes were markedly increased.\textsuperscript{90} It appears that excess ADMA can alter endothelial function both dependently and independently of NOS. The effects of excess ADMA further highlight the importance of understanding how PRMTs are regulated, how they become dysregulated, and what can be done to prevent or reverse these negative physiological consequences.

A third area of clinical significance for PRMTs is spinal muscular atrophy (SMA). SMA is an autosomal recessive genetic disorder resulting from deletions in the survival motor neuron (SMN) gene.\textsuperscript{91} SMN proteins contain Tudor domains, which are known to interact with other proteins in a methylation-dependent manner.\textsuperscript{92–94} These SMN-interacting proteins include substrates of PRMTs 4 and 5, making both ADMA and SDMA important post-translational modifications linked with the progression of SMA. Some of these same SDMA-modified protein substrates have also been linked with systemic lupus erythematosus (SLE).\textsuperscript{95} Understanding how these PRMTs are regulated and being able to artificially manipulate their activity could provide methods of alleviating symptoms and increasing quality of life for patients with SMA and SLE.

A fourth and final clinically significant aspect of PRMTs discussed here is viral pathogenesis. PRMTs 1, 5, and 6 and their methylated substrates have been linked to the virulence of HIV, Epstein-Barr virus (EBV), and hepatitis delta virus (HDV).\textsuperscript{96–98} In HIV, PRMT6 methylation of a Tat protein resulted in a negative regulatory effect by
diminishing Tat’s affinity for RNA and other proteins. This suggests that activation of PRMT6 could help decrease the virulence of HIV. One of the very first proteins made by the EBV upon infection (EBNA2) is methylated by PRMT5. This methylation is required for protein-protein interactions that allow B cell transformation and progression of infection. In HDV, the methylation of a small hepatitis delta antigen by PRMT1 is essential for the replication of RNA, while inhibiting this methylation event blocks viral replication. These findings suggest that specifically blocking or enhancing certain methylation events could help treat or slow the progression of these viral infections.

These examples emphasize the urgency that exists in learning more about PRMTs and their influence in disease. Considering the widespread and important role that PRMTs play in healthy and unhealthy cells, it becomes even more important to discover how PRMTs are regulated, and how we can exploit their regulatory mechanisms in treating and managing the above described human diseases. Working within a yeast model allows a more tractable approach while still providing valuable insight into the regulation of the PRMT family. This thesis focuses on the yeast protein Pop2, and its potential role as a regulator of the yeast homolog of PRMT1, Hmt1.

**Pop2: A Potential Regulator of PRMTs**

hCaf1 was discussed earlier as a novel regulator of PRMT1 activity with respect to certain substrates. Although hCaf1’s regulatory mechanism is still unclear, this finding provided a starting point to explore this phenomenon in the eukaryotic yeast model, *Saccharomyces cerevisiae*. Pop2, similar to its human homolog hCaf1, is part of the Ccr4-Not complex, and possesses deadenylase activity both with its complex and alone. Although Pop2 is capable of deadenylation, the main deadenylase of the Ccr4-Not complex is Ccr4. Pop2 does, however, play a unique regulatory role by
interacting with other proteins such as Puf5 in recruiting the rest of the complex to mRNAs.\textsuperscript{102}

The Ccr4-Not complex regulates gene products almost every step of the way from transcription and mRNA nuclear surveillance to nuclear export, translational quality control, deadenylation and mRNA decay, and thus resides in both the nucleus and the cytoplasm.\textsuperscript{101,103–105} Knocking out each of the nine subunits of the Ccr4-Not complex one at a time resulted in altered mRNA transcript levels for many genes, emphasizing this complex’s role as a general regulator of transcription and mRNA decay.\textsuperscript{106} The Ccr4-Not complex has also been shown to associate with the proteasome, and together, regulate lysine methylation modifications on histones in a ubiquitin-dependent manner.\textsuperscript{107} If the Ccr4-Not complex regulates lysine methylation, then it is feasible that the complex or one of its components could also regulate arginine methylation. Indeed, this was shown in the PRMT1 and hCaf1 study mentioned earlier. In yeast, several components of the Ccr4-Not complex (including Pop2) were demonstrated to interact with Hmt1 and several of Hmt1’s substrates.\textsuperscript{105} Considering the relative novelty of these findings, very few details about the interactions and regulatory mechanisms of hCaf1 and Pop2 (in addition to the rest of the Ccr4-Not complex) and their roles in arginine methylation have been elucidated. The studies presented in this thesis begin to uncover the details in a yeast model system of Hmt1 regulation by Pop2 in order to better understand how hCaf1 regulates PRMT1 in humans and therefore advance our understanding of PRMT regulation.

**Summary and Hypothesis**

Protein arginine methylation is a vital and widespread PTM conserved throughout eukaryotic systems. PRMTs and their substrates play a significant role in a variety of
biological processes and many human diseases, including cardiovascular disease and cancer. Understanding how PRMTs are regulated is an important step in utilizing medicinal tools in treating and curing these diseases, but there is still a great deal of progress that needs to be made before we can fully unlock the potential of modulating PRMT activity. Recent evidence suggests that hCaf1 in humans, and Pop2 in yeast, may help illuminate some of the regulatory mechanisms of PRMT1 and Hmt1, respectively. In order to contribute to this ever-growing and significant area of research, this thesis will provide part of the foundation needed to understand Hmt1 regulation by Pop2. Chapter 2 describes the optimized methods used to express and purify the yeast Pop2 protein. Chapter 3 presents data that corroborates and expounds upon the homologous function of hCaf1/Pop2 arginine methylation regulation and proposes a regulatory mechanism based on novel protein-protein interactions. These studies, combined with future research, will continue to expand our knowledge of the methylproteome and its regulatory mechanisms, ultimately providing the basis for the future treatment and prevention of many prominent human diseases.

References


CHAPTER 2
EXPRESSION AND PURIFICATION OF POP2 PROTEIN

Introduction

Protein arginine methylation is an important post-translational modification (PTM) that is vital in regulating various cellular processes such as gene transcription, cell signaling, and RNA processing.\(^1\)\(^-\)\(^5\) PRMTs (protein arginine methyltransferases) are enzymes that perform this modification. This family of enzymes utilizes a methyl donor called S-adenosyl-L-methionine, abbreviated as SAM or AdoMet. The methyl group is transferred from SAM to a terminal guanidino nitrogen on a protein arginine residue to form MMA (monomethyl arginine). The substrate can then be further methylated to form ADMA (asymmetric dimethyl arginine), which is catalyzed by type I PRMTs, or SDMA (symmetric dimethyl arginine), which is catalyzed by type II PRMTs. A PRMT that stops at the production of only MMA is considered a type III PRMT.\(^5\)\(^,\)\(^6\) PRMT1 is the predominant arginine methyltransferase in mammals, and catalyzes the formation of ADMA, making it a type I PRMT.\(^7\)

The study of PRMTs has gained momentum in recent years as their roles in human diseases have become more apparent. In fact, PRMTs have been implicated in cardiovascular disease, spinal muscular atrophy, viral pathogenesis, and cancer.\(^4\)\(^,\)\(^5\) As such, PRMTs are becoming a more prominent target for the development of pharmaceuticals to help treat or even cure the human diseases that are related to this PTM.\(^8\) However, before we can manipulate or alter the activity of malfunctioning or dysregulated PRMTs, there is much that needs to be discovered about how arginine methylation is regulated in the cell. Despite the structural, kinetic, and catalytic characterization of many of the PRMTs, relatively little is known about their regulation.
Since no true arginine demethylases have been discovered, the regulation of this somewhat permanent PTM becomes even more crucial to the cell. The currently known methods of PRMT regulation include direct modification of PRMTs with PTMs, oligimerization, deimination, adjacent PTMs of substrates, and PRMT-binding proteins and small molecules.\textsuperscript{1,5,8–13} Of particular interest to us is the regulation of arginine methylation via protein-protein interactions.

A certain human protein called hCaf1 was discovered to be a novel regulator of PRMT1 with respect to certain substrates.\textsuperscript{9} Here, preliminary data suggest that the yeast homolog of hCaf1, Pop2, may also perform a similar function on Hmt1. However, in order to explore this novel regulator of Hmt1, the expression and purification conditions needed to be optimized. The final construct that expressed and purified most successfully was a truncation (Δ1-146) of Pop2, similar to the one used to solve the crystal structure of Pop2.\textsuperscript{14} This construct also included a TEV (tobacco etch virus) cleavable N-terminal polyhistidine tag (His-tag), and the protocols for purifying this protein required slight but critical variations to standard purification methods.

**Materials and Methods**

*Materials* – QIAprep Spin Miniprep kit was purchased from Qiagen. Nickel resin was purchased from GE Healthcare. SAM was purchased from Sigma. $[^3\text{H}]-\text{SAM}$ was purchased from Perkin Elmer, and had a specific activity of 83.1 Ci/mmol. Histone H4 protein was purchased from New England Biolabs. R3 peptide is a short peptide substrate commonly used for arginine methylation and has the following sequence: GGRGGFGRGGFGGRGGGG. ZipTips®\textsubscript{C\text{18}} pipette tips were purchased from Millipore.
Molecular Cloning – The Pop2 gene (accession number P39008) was PCR amplified from S. cerevisiae cDNA using primers with NdeI and BamH1 restriction sites. Full length Pop2 (433 aa) was inserted into a pET28b(+) vector with an N-terminal His-tag. The truncated (Δ1-146) Pop2 was similarly PCR amplified and inserted into a modified pET28b(+) vector that also contained a TEV-cleavage site between the His-tag and the protein sequence. Additional vectors included a pDR177-Strep(II) expression vector, based on a pET15b vector, as well as a pDB-GST PSI vector. All plasmids were transformed into E. coli DH5α cells, grown overnight in 2XYT growth media at 37°C, and purified using a QIAprep Spin Miniprep Kit. All plasmids were sent for sequencing to verify correct sequence, reading frame, etc.

Recombinant Protein Expression – The plasmids with their respective Pop2 constructs were transformed into E. coli BL21 codon plus (DE3) cells in the presence of appropriate antibiotics. Cells were grown in 2XYT media at 37°C until OD600 reached 0.6-0.8, followed by induction with 0.5mM IPTG. Induced cell growth continued at room temperature for 24 hours. Cells were harvested via centrifugation and stored at -80°C until used for purification of Pop2.

Protein Purification of Full Length Pop2 and Truncated (Δ1-146) Pop2 – Cell pellets were resuspended in three times cell volume lysis buffer (50 mM phosphate, 100 mM NaCl, 20 mM imidazole, 5 mM MgCl2, 10 μg/mL RNase A, 10 mM β-mercaptoethanol, pH=7.5). Cells were lysed using sonication, and cell debris was pelleted using centrifugation. Clarified supernatants were applied to equilibrated nickel resin in a 5:1 cell mass: resin ratio and allowed to incubate for 2 hours at 4°C. Resin was washed eight times with ten column volumes of wash buffer (50 mM phosphate, 100 mM NaCl, 20 mM imidazole, 5 mM MgCl2, pH=7.5), followed by six elutions with two column volumes of elution buffer (50 mM phosphate, 100 mM NaCl, 250 mM imidazole, 5 mM
MgCl₂, pH=7.5). Elutions were pooled and filtered, and imidazole was eliminated by two rounds of dialysis (two liters each of 50 mM phosphate, 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, 10 mM β-mercaptoethanol, pH=7.5). At this point, full length Pop2 was beaded in liquid nitrogen and stored at -80°C. Truncated (Δ1-146) Pop2 was incubated overnight to TEV cleave the histidine tag at 16°C. Protein was then put back over nickel resin to bind TEV, incubated at 16°C, and Pop2 (Δ1-146) was collected in the flow through. Purified protein was then beaded in liquid nitrogen and stored at -80°C.

**Enzyme Assays** – Reactions were performed in a 30°C water bath with various concentrations of Pop2, 100 nM Hmt1 enzyme, 10 nM MTAN, 0.38 μM BSA, and 2 μM SAM (1 μM cold SAM and 1 μM [³H]-SAM), in 50 mM phosphate buffer. Substrates were either 0.5 μM histone H4 protein substrate, or 200 μM R3 peptide. Time points were taken and quenched in 6 M guanidinium hydrochloride. Samples were processed using ZipTips® C18, which separated unreacted [³H]-SAM from radioactively labeled product. Processed samples were analyzed using a scintillation counter to quantify the amount of methylation.

**Results and Discussion**

The first construct of Pop2 from *S. cerevisiae* that was attempted was a full length His-tagged Pop2 construct. Despite multiple attempts with various growth conditions and cell lines, Pop2 expression was considerably low. In addition, there were two persistent contaminants (approximately 60 and 75 kDa) that were very difficult to eliminate (Figure 2-1). Despite the poor expression levels and less than ideal purity of Pop2, preliminary assays were performed to see if Pop2 had any regulatory effect on the methylation of histone H4 protein substrate. Hmt1 reactions using [³H]-SAM were used to measure the amount of methylation in the presence and absence of Pop2 (Figure 2-
2). These preliminary results suggested that Pop2 does inhibit the methylation of histone H4 protein by Hmt1. This prompted us to move forward in optimizing the expression and purification of Pop2 for further assays as well as other experiments for better understanding the interactions and inhibitory mechanism of Pop2.

**Figure 2-1. Nickel resin purification of full-length His-tagged Pop2 protein.** Pel = pellet. Sup = supernatant. FT = resin flow through following incubation. Pop2 is outlined in black box.

**Figure 2-2. Preliminary Hmt1 enzyme assay showing methylation inhibition by Pop2.** Reactions were performed with histone H4 protein substrate in the presence (○) and absence (■) of full length His-tagged Pop2 protein. No substrate present was used as a baseline (●).
Pop2 was inserted into other vectors in an attempt to improve total protein yield and purity of final product. These included Streptavidin-tagged Pop2, and GST-TEV-Pop2 constructs. Streptavidin-Pop2 was unsuccessful because of such poor expression, as well as the persistence of the same two impurities from the first construct (Figure 2-3). Additionally, a buffer condition that would TEV cleave the Streptavidin tag could not be determined. As a result, this construct was abandoned in an attempt to find a construct of Pop2 with better expression and purification results.

Figure 2-3. Streptavidin resin purification of full-length Strep-TEV-Pop2 construct. Pop2 protein is outlined in black box. P = pellet. S = supernatant. FT = flow through. W = washes.

The next construct attempted was a TEV-cleavable GST-tagged full-length Pop2. The GST-TEV-Pop2 construct expression was slightly better, but ultimately did not work because a condition to TEV-cleave the GST tag could not be identified. Multiple buffers and conditions were attempted with TEV, without success. Notwithstanding this, inhibition assays similar to the ones described for the full length Pop2 were performed with the GST-tagged Pop2 to see if the uncleaved protein would still show signs of methylation inhibition. However, there was no apparent inhibition of histone H4 methylation, making this uncleaved construct unviable for future experiments. We
hypothesized that the GST tag was obstructing an essential part of Pop2 that interfered with its ability to regulate methylation. Similar to the Strep-Pop2 construct, this construct was also abandoned and the search continued for an expressible, TEV-cleavable Pop2 protein that maintained its inhibitory effect on substrate methylation by Hmt1.

The last Pop2 construct attempted left out the first 146 amino acids from the N-terminus of the protein sequence, thus creating a similar construct to that used to solve the crystal structure of Pop2.\textsuperscript{14} In addition to matching the crystal structure construct, another reason for choosing this truncation was its sequence conservation with hCaf1. The N-terminus of Pop2 does not align with hCaf1; sequence and structural alignments with these two proteins are in the RNase domain, which begins at residue 147 for Pop2.\textsuperscript{14}

The Pop2 (Δ1-146) truncation was inserted into a modified pET28b(+) vector that included a His-tag followed by a TEV protease cleavage site. Expression of the protein was greatly increased for this construct, and the purity was also markedly improved (Figure 2-4 A). Once Pop2 (Δ1-146) was purified, the next hurdle was TEV-cleaving the His-tag. Surprisingly, the tag could be cleaved relatively easily under standard conditions. However, severe precipitation would occur following TEV cleavage, resulting in almost complete loss of any soluble protein.

All attempts to TEV-cleave the His-tag were done at 4°C in order to preserve the integrity and catalytic activity of the proteins. However, closer inspection of the Pop2 crystal structure paper reported TEV cleavage at 16°C.\textsuperscript{14} When the TEV cleaving step was performed at this higher temperature, the cleaved sample contained little to no precipitation, resulting in much greater total protein yielded after the filtration step (Figure 2-4 B). Cleaved protein was put back over nickel resin to remove TEV protease enzyme and other impurities that non-specifically bound to nickel resin. This step also needed to
be carried out at 16°C to avoid protein precipitation. Cleaved Pop2 (Δ1-146) protein was collected in the filtered unbound fraction, indicating that Pop2 (Δ1-146) was still soluble and stable in solution.

![Diagram](image.png)

**Figure 2-4. Nickel resin purification of His-TEV-(Δ1-146) Pop2 and TEV cleavage.** (A) Nickel resin purification. P = pellet. S = supernatant. FT = flow through. Purified Pop2 protein is outlined in black box. (B) His-TEV-(Δ1-146) Pop2 TEV cleavage and repurification using nickel resin. Cut = TEV cleaved protein. FT = nickel resin flow through following incubation to bind TEV protease to resin. Filt. = filtered Pop2 protein. Purified Pop2 protein is outlined in black box.

**Conclusion**

After numerous attempts to express and purify the Pop2 protein, the most successful construct purified was a truncated construct consisting of amino acids 147-433. Standard growth and His-tag purification procedures were utilized during purification of Pop2 (Δ1-146). Interestingly, the TEV cleaving step and the subsequent purification step to remove TEV required a 16°C incubation temperature to avoid severe protein precipitation. A 16°C TEV-cleavage and repeated nickel purification to bind TEV protease and other impurities was much more successful and yielded a much higher amount of protein than the same procedure at 4°C. This suggests that the truncated (Δ1-146) Pop2 construct is a temperature sensitive protein.
Once the Pop2 (Δ1-146) protein expression and purification protocol was optimized, a substantial amount (several milligrams) of the purified protein was able to be stored for later use in experiments designed to further our understanding of Hmt1 methylation regulation. Chapter 3 provides evidence of inhibition of Hmt1 activity by the truncated (Δ1-146) Pop2 construct, and explores the scope of regulation by full length and truncated (Δ1-146) Pop2 on a variety of Hmt1 substrates. Chapter 3 also investigates the mechanism that Pop2 (Δ1-146) utilizes to accomplish this regulation, and analyzes the role of specific residues on the surface of Pop2 (Δ1-146) to probe a potential binding interface responsible for this regulatory function.

References


CHAPTER 3
REGULATION OF HMT1-CATALYZED METHYLATION BY POP2

Introduction

The expansion of the proteome relies on post translational modifications (PTMs) to alter protein function. Arginine methylation is an important and widespread PTM essential for a variety of cellular processes, including transcriptional regulation, RNA processing, signal transduction, and others. The methylation of arginine residues relies on a family of enzymes called PRMTs (protein arginine methyltransferases) to perform this modification. PRMTs require a methyl group donor, S-adenosyl-L-methionine, abbreviated as SAM or AdoMet. The methyl group is transferred from SAM to a terminal guanidino nitrogen of an arginine residue in the protein substrate, forming S-adenosyl-L-homocysteine (SAH, or AdoHcy) and methylated arginine as products. Given its unique structure, arginine can be monomethylated (MMA), asymmetrically dimethylated (ADMA), or symmetrically dimethylated (SDMA).

PRMTs are classified based on which form of methylated arginine they produce. Type I PRMTs produce MMA and ADMA; type II PRMTs produce MMA and SDMA; type III PRMTs produce solely MMA.1,2 Each of these modifications is unique and can produce different biological effects. PRMTs have received greater attention in recent years as these biological consequences have been implicated in human diseases such as cardiovascular disease, cancer, viral pathogenesis, and spinal muscular atrophy.1–6 Given the growing evidence of the roles of PRMTs in disease, they are increasingly becoming a target for therapeutic pharmaceuticals.7 However, there is much to learn about PRMTs and how they are regulated before they can be specifically manipulated or altered to treat such diseases.
Compared to the global knowledge of PRMTs, relatively little is understood about their regulation. We know of several mechanisms for regulating PRMTs, such as direct modifications of PRMTs, adjacent PTMs on substrates, deimination, protein-protein interactions, as well as others.\textsuperscript{1,8–12} However, this knowledge is just the beginning of what is becoming a diverse and complex network of regulatory mechanisms for PRMTs.

PRMT1 is the predominant PRMT in mammals and catalyzes the formation of MMA and ADMA, classifying it as type I.\textsuperscript{13} As the workhorse of the PRMT family, it is consequently the most well understood PRMT with numerous studies investigating its mechanism, structure, substrate specificity, and regulation. One such regulator, a human protein called hCaf1 (human Ccr4-associated factor 1), was discovered to regulate the methylation of certain substrates by PRMT1, specifically, Sam68 and histone H4.\textsuperscript{14} hCaf1 and PRMT1 were also shown to colocalize \textit{in vivo}, suggesting they could be interacting directly, or perhaps indirectly through one or more bridging molecules.\textsuperscript{14} This piqued our interest in discovering how PRMT1 could be regulated by this protein. However, in order to take a closer look at how human PRMTs are regulated, it is often good to start in a simpler model such as \textit{Saccharomyces cerevisiae}, or baker's yeast.

Hmt1 is the yeast homolog of PRMT1, making it a type I PRMT, and the predominant methyltransferase in \textit{S. cerevisiae}.\textsuperscript{12} The yeast homolog of hCaf1 is Pop2, and these two proteins share many characteristics as they are both deadenylases in the Ccr4-Not complex.\textsuperscript{15} Other encouraging findings included the discovered association of Hmt1 and Pop2 in yeast, as well as the potential regulation of histone lysine methylation by members of the Ccr4-Not complex.\textsuperscript{16,17} This led us to hypothesize that Pop2, like hCaf1, may also regulate the methylation of certain Hmt1 substrates, such as histone H4 protein.
Materials and Methods

*Molecular Cloning* – Pop constructs and vectors were obtained and utilized as described in Chapter 2. The *S. pombe* plasmid used was a pET-30 Ekl/LIC with a TEV cleavage site between the His-tag and the protein sequence. It was generously sent to us as a gift from Dr. Ditlev Broderson, Department of Molecular Biology and Genetics, Aarhus University, Denmark. *S. cerevisiae* Pop2 (Δ1-146) variants (E260A, E263A, and K267A) were created using appropriately designed primers and a QuikChange Lightning Site Directed Mutagenesis Kit (Agilent Technologies). All plasmids were sent for sequencing to verify correct mutations, sequence, and reading frame.

*Protein Expression and Purification* – Full-length and truncated (Δ1-146) Pop2 from *S. cerevisiae* were expressed and purified as described in Chapter 2. Pop2 from *S. pombe* was expressed using BL21 Ni-Co (DE3) cells in ZY media, and protein expression was induced using auto-induction. Cell pellet was resuspended in two cell volumes of lysis buffer (50 mM Tris, 200 mM KCl, 20 mM imidazole, 5 mM MgCl₂, 20% glycerol, pH 8.0). Cells were lysed using sonication, and centrifuged to collect supernatant. *S. pombe* Pop2 was expressed with a His-tag, which allowed nickel resin purification. Supernatant was incubated for 1.5 hours with nickel resin equilibrated with lysis buffer. Nickel resin was washed 10 times with wash buffer (50 mM Tris, 200 mM KCl, 20 mM imidazole, 5 mM MgCl₂, 3 mM BME, pH 8.0) followed by 7 elutions with elution buffer (50 mM Tris, 200 mM KCl, 250 mM imidazole, 5 mM MgCl₂, 3 mM BME, pH 8.0). Elutions were pooled, filtered, and allowed to dialyze with TEV protease (100:1 ratio) overnight at 4°C in dialysis buffer (50 mM Tris, 100 mM KCl, 5 mM MgCl₂, 3 mM BME, pH 8.0) to remove the His tag. TEV-cleaved protein was put back over nickel resin to bind the TEV protease enzyme, and the purified protein sample was collected and stored at -80°C.
**Enzymatic Assays** – Reactions were carried out as described in Chapter 2. Protein substrate concentrations (Npl3, histone H4 protein) were 0.5 μM. Peptide substrate concentrations were 200 μM. Enzyme concentration (Hmt1) remained at 100 nM for all reactions. All other concentrations are as listed in Chapter 2.

**Yeast Cell Growth and Lysate Preparation** – The yeast strains used (wild type, ΔPop2, and ΔHmt1) were generously provided by Dr. Michael C. Yu, Department of Biological Sciences, University at Buffalo, New York. Yeast cells were grown in YEPD media to an OD$_{600}$ of 1.0, then washed with PBS and treated with various media conditions for 2 hours: YEPD, YEP (no glucose added), or YEPD media with 200 nM Rapamycin. Cells were harvested via centrifugation. Pellets were resuspended in approximately 20 cell volumes of lysis buffer (PBS, 10 μg/mL RNase A, 1 mM EDTA, 1 mM PMSF), and sonicated in a Rosette cell to provide optimal cooling conditions while thoroughly lysing the cells. Cell lysates were clarified with centrifugation, and supernatants were collected.

**Western Blot Analysis** – Western transferring and blotting were performed according to manufacturer’s instructions (BioRad) using a PVDF membrane. Antibodies used were as follows: Rabbit polyclonal to beta actin – loading control (Abcam ab8227-50); Anti-asymmetric dimethyl arginine ASYM24 (Upstate Cell Signaling Solutions); Mouse monoclonal to mono and dimethyl arginine (Abcam ab412-200). Western blots were developed using Pierce Fast Western Blot Kit (Thermo Scientific) detection reagents, and visualized with a Fuji LAS-3000 Lite imager.

**Radioactive Gel Assay** – Reactions were performed similarly to those mentioned above. Sample time points were quenched in XT Sample Buffer (BioRad), and run on Criterion XT Bis-Tris 10% Acrylamide Precast Gel according to manufacturer’s instructions (BioRad). The gel was transferred to a PVDF membrane using standard
Western blotting protocol. The membrane was sprayed with En3Hance Spray (Perkin Elmer) and exposed to film. Film was developed and imaged using Fuji LAS-3000 Lite imager.

Results and Discussion

As discussed in Chapter 2, full-length Pop2 from *S. cerevisiae* was shown to inhibit the methylation of histone H4 protein by Hmt1 enzyme. However, due to low expression and poor purification of the full-length Pop2, a truncation with a TEV-cleavable His-tag was used for additional experiments. This construct included amino acids 147-433, which is the RNase domain and the portion of the Pop2 sequence that is conserved with the human hCaf1 protein.18 We hypothesized that the N-terminus of Pop2 would not play a role in its regulation of PRMTs since hCaf1 does not contain this domain. Indeed, radioactive assays demonstrated that the Pop2 (Δ1-146) construct inhibits the methylation of histone H4 protein by Hmt1 in a concentration-dependent manner (Figure 3-1). With increasing concentrations of Pop2 (Δ1-146), we observed a linear decrease in overall rate of the reaction. With respect to histone H4 protein as a substrate, Pop2 (Δ1-146) does appear to regulate methylation by Hmt1 similarly to hCaf1.

The next step was investigating the effect of Pop2 on the methylation of other substrates, both proteins and peptides. This would help us decipher the inhibitory mechanism of Pop2, as well as its specificity as a regulator. Among other Hmt1 substrates tested were Npl3, a yeast hnRNP protein, and R3, a common 20-residue *in vitro* peptide substrate used in PRMT enzymatic activity assays. However, the methylation rate of neither of these substrates seemed to be significantly affected by the presence of Pop2 (Figure 3-2). This suggested that Pop2 is able to specifically regulate
Figure 3-1. Pop2 (Δ1-146) concentration-dependent inhibition of Hmt1-catalyzed methylation of histone H4 protein substrate. (A) Quantified methylation at various time points for reactions containing no Pop2 (■), 50 nM Pop2 (♦), 200 nM Pop2 (▲), 350 nM Pop2 (▼), and no substrate as the baseline control (●). (B) Reaction rates from A plotted against concentration of Pop2 (Δ1-146), showing the linear decrease in reaction rate with increasing concentrations of Pop2 (Δ1-146).

Figure 3-2. Full-length and truncated (Δ1-146) Pop2 inhibition assays of Hmt1-catalyzed methylation of various substrates. (A) Methylation of Npl3 protein substrate at various time points for reactions containing no Pop2 (■), and 100 nM full-length Pop2. Reactions were initiated with either substrate (♦), or with enzyme (▲). No substrate was used as the baseline control (●). (B) Similar to A, reaction time points with R3 peptide substrate without Pop2 (■), or with 100 nM Pop2 (Δ1-146) (♦), and the absence of substrate as the baseline control (●).
the methylation of histone H4 protein substrate and possibly other substrates, while leaving others unaffected.

In order to investigate the conservation of the regulatory function exhibited by Pop2 among yeast species, activity assays with histone H4 protein as substrate were also performed with Pop2 from *Schizosaccharomyces pombe* (Figure 3-3). We hypothesized that Pop2 from this yeast species would also exhibit a similar regulatory effect. As expected, there was a significant drop in methylation of histone H4 protein substrate in the presence of *S. pombe* Pop2 in addition to full-length *S. cerevisiae* Pop2. Not only does this result suggest an evolutionarily conserved function for Pop2 in the regulation of Hmt1-catalyzed methylation in yeast, it also reaffirms the evolutionary relationship between hCaf1 and both yeast species of Pop2 in regulating arginine methylation of certain substrates. This implies a conservation of an important regulatory mechanism throughout eukaryotic evolution.

![Figure 3-3](image)

**Figure 3-3.** Full-Length Pop2 from *S. cerevisiae* and *S. pombe* inhibition assays of Hmt1-catalyzed methylation of histone H4 protein substrate. Methylation of histone H4 protein substrate at various time points for reactions containing no Pop2 (■), 100 nM full-length His-tagged *S. cerevisiae* Pop2 (♦), or 100 nM full-length *S. pombe* Pop2 (▲). A reaction with no substrate present was used as the baseline control (●).
Our next goal was to explore the extent of the effects of Pop2 on other substrates in a broader context. In order to accomplish this, several experiments were done with whole yeast cell lysates. The first experiment involved reactions with hypomethylated yeast cell lysate obtained from ΔHmt1 yeast cells. The lysate was used in reactions with \[^{3}\text{H}]-\text{SAM}\) in the presence and absence of added full-length His-tagged Pop2, followed by western transfer and exposure to film to visualize protein bands that had incorporated tritium. In this experiment, the presence of full-length Pop2 demonstrated an altered pattern of methylation of multiple yeast protein substrates, suggesting a broader scope of influence for Pop2 in PRMT regulation (Figure 3-4 A).

![Image](image.png)

**Figure 3-4. Pop2 from *S. cerevisiae* and its effects on yeast cell lysate methylation.** (A) Autoradiograph of reactions with hypomethylated yeast cell lysate from ΔHmt1 yeast cells +/- Pop2 in the presence of \[^{3}\text{H}]-\text{SAM}\) and exogenous Hmt1. Time points were taken at 20 and 60 minutes. –Sub: no yeast cell lysate (control). –Pop2: no Pop2 added. +Pop2: 100 nM Pop2 added. (B) Western blot of wild type (WT) and ΔPop2 (ΔP) yeast cells grown in various media: regular media (YEPD), no glucose (YEP), and YEPD +200 nM Rapamycin (Rap). Top panel: ADMA antibody. Middle panel: MMA/ADMA antibody. Bottom panel: Beta-actin loading control antibody.

This finding was further validated in another experiment involving wild type and ΔPop2 yeast strains grown in various media conditions: normal media (YEPD), glucose deprived (YEP), and YEPD media with 200 nM Rapamycin. Rapamycin is a drug...
commonly used to imitate a cell starvation response. These conditions were chosen because of studies that reported the phosphorylation of Pop2 under glucose limiting conditions, and altered Hmt1 activity under similar starvation or Rapamycin-treated conditions.\textsuperscript{9,19} We were interested in the effects these conditions would have on Pop2 and whether they would alter its regulatory function. Western blot analysis was used to compare the two different yeast strains from each type of media, and demonstrated that the absence of Pop2 led to an increase in the amount of methylation in all tested media conditions, including glucose deprivation (Figure 3-4 B). This again suggested that Pop2 may regulate the methylation of many substrates beyond just histone H4 protein, and could be involved in a variety of regulatory pathways.

The next major question that needed to be addressed was how Pop2 was able to accomplish its regulatory effect on arginine methylation. Previous studies have shown Hmt1 and Pop2 to associate, and PRMT1 and hCaf1 have been shown to colocalize.\textsuperscript{14,16} Our first hypothesis was that Pop2 and Hmt1 could be directly interacting. However, after numerous attempts to detect a complex between these two proteins using cross-linking, pull-downs, and immunoprecipitation experiments, no complex was observed (pull-down in Figure 3-5 A, other data not shown). A transient complex could have formed with weak interactions, but we could not detect or isolate it. The next hypothesis tested was that Pop2 could be interacting with the substrate instead of the enzyme, and is thus able to accomplish its selective inhibition. Control pull-downs with Pop2 (Δ1-146) and histone H4 protein alone revealed that histone H4 protein nonspecifically binds to nickel resin very tightly (data not shown). We were thus able to use histone H4 protein to pull down TEV-cleaved Pop2 (Δ1-146), which was no longer capable of binding to nickel
resin. These pull-downs using purified histone H4 protein and Pop2 (Δ1-146) suggested a direct interaction as the two proteins eluted together (Figure 3-5 B).

This interaction between Pop2 (Δ1-146) and histone H4 protein was an exciting discovery because it suggests a novel protein-protein interaction that has not previously been postulated. It was known that Pop2 and hCaf1 associated with their respective PRMTs, but their association was not known to be via direct interactions or not. This novel interaction between Pop2 and histone H4 protein suggests that histone H4 (and possibly other proteins as well) could potentially act as a bridging molecule that causes Pop2 and Hmt1 to be associated in turn.

In order to identify the specific surface of Pop2 (Δ1-146) responsible for its interactions with histone H4 protein, several Pop2 (Δ1-146) variants were designed based on a solvent-exposed helix from the crystal structure (Figure 3-6). This particular helix was hypothesized as a potential site of interaction for several reasons. First, some of the other conserved surfaces of Pop2 are already known to interact with other
proteins, such as Ccr4 and Not1 of the Ccr4-Not complex via hydrophobic interactions (Figure 3-6).\textsuperscript{20,21} These surfaces were eliminated as they were less likely to be responsible for the novel interaction with histone H4 protein. Second, the outward-facing residues of this helix were conserved with \textit{S. pombe} Pop2 and with hCaf1. If there is a specific function conserved among these proteins, then it usually follows that there are structural and sequential characteristics that are also conserved. Based on this reasoning, we hypothesized that there must be some commonality between these three Pop2 homologs which allowed them to achieve their regulatory function. We further hypothesized that the selected helix or the region surrounding it could be part of that commonality responsible for methylation inhibition.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-6.png}
\caption{\textbf{Figure 3-6. Crystal structure of \textit{S. cerevisiae} Pop2 (Δ1-146).} Backbone of Pop2 is shown in blue. Locations of interactions with two other members of the Ccr4-Not complex, Ccr4 and Not1, are labeled as shown. Ccr4 is located diagonally down and to the left. Not1 is located to the right and towards the back of the structure. The residues chosen for mutagenesis are labeled in orange with side-chains shown as sticks. All three residues shown in orange were mutated to alanine.}
\end{figure}
Three outward-facing conserved residues on the selected helix of Pop2 (Δ1-146) were chosen for mutagenesis: E260, E263, and K267 (Figure 3-6). Each of these residues were mutated to alanine, and the wild type Pop2 (Δ1-146) truncation was used as the template.

Inhibition assays were performed with wild type Pop2 (Δ1-146), and each of its variants (E260A, E263A, and K267A), similar to the assays discussed earlier (Figure 3-7). Both E260A and E263A showed similar inhibitory effects to wild type Pop2 (Δ1-146). However, the K267A variant had a decreased ability to inhibit methylation. This led us to hypothesize that K267 may be an important residue for interacting with histone H4 protein. Additional experiments will further probe the precise surfaces of Pop2 that are involved in this regulatory protein-protein interaction.

![Figure 3-7](image)

Figure 3-7. Methylation assays with Hmt1 and histone H4 protein substrate in the presence and absence of Pop2 (Δ1-146) wild type and mutants. (A) Hmt1 reactions with [3H]-SAM similar to those previously described. Reactions with no Pop2 (Δ1-146) (●), wild type (■), E260A (▲), E263A (▼), and K267A (♦) time points are shown. (B) Methylation reaction rates from panel A plotted for each type of Pop2 (Δ1-146): wild type (WT), and the three variants.
The next step was to see if the decreased inhibitory capacity of Pop2 (Δ1-146) K267A was due to a hindered ability to interact with histone H4 protein. In order to assess this, nickel resin pull-downs were performed with histone H4 protein similarly to those performed with wild type Pop2 (Δ1-146) (Figure 3-8). As the figure shows, Pop2 (Δ1-146) K267A eluted with histone H4 protein, suggesting that the two proteins are still interacting. That being said, this experiment was a more qualitative method of examining the interaction between these two proteins. Future experiments using more quantitative techniques can investigate more slight alterations in the amounts of interacting protein to test whether Pop2 (Δ1-146) K267A has a lowered affinity for histone H4 protein.

![Figure 3-8. Nickel resin pull-down with Pop2 (Δ1-146) K267A.](image)

**Figure 3-8. Nickel resin pull-down with Pop2 (Δ1-146) K267A.** TEV-cleaved Pop2 (Δ1-146) K267A and histone H4 protein were incubated together, followed by nickel resin pull-downs utilizing the nonspecific binding of histone H4 protein to nickel resin.

**Conclusion**

We have demonstrated concentration-dependent methylation inhibition of histone H4 protein substrate by Pop2 (Δ1-146) protein from *S. cerevisiae*, and show this regulatory effect to be conserved in *S. pombe*. We also present data that shows an altered methylation pattern of many other yeast substrates *in vivo* and *in vitro* in the presence and absence of Pop2.
Based on a novel interaction discovered between Pop2 (Δ1-146) and histone H4 protein, we propose a substrate-sequestering mechanism that accomplishes methylation inhibition of histone H4 protein substrate. This was a fascinating and rather unexpected discovery, since many regulators bind to the enzyme rather than the substrate to regulate catalytic activity. A diminished ability to inhibit methylation by the K267A variant of Pop2 (Δ1-146) led us to hypothesize that the region around this residue may be involved. This finding could have powerful significance if the same regulatory mechanism exists in humans with hCaf1, and if the interaction with histone H4 can be specifically altered or modified for medicinal applications.

This and future experiments will further expound upon the details of how PRMTs are regulated in vivo in yeast and mammals and how they can be pharmaceutically regulated. Given the wide variety of arginine methylation substrates, artificial regulation of PRMT activity toward specific substrates is an extremely difficult challenge. However, with these findings and other future work, there is potential for the design of substrate-specific regulators of PRMTs, which would allow selective methylation inhibition of certain PRMT targets while leaving other substrates unaffected. Accomplishing this goal of selective PRMT regulation could have profound implications in the treatment of the many human diseases in which PRMTs have been implicated so far.

References


CHAPTER 4
SUMMARY AND FUTURE WORK

Review

Eukaryotes have a multitude of cellular pathways and processes that must be maintained for survival, from the simpler organisms like unicellular yeast to the much more complex human. In order to accomplish such a diverse set of functions, the proteome has the capability to be modified and expanded through post translational modifications (PTMs). One of these critical PTM’s is arginine methylation, which is catalyzed by the protein arginine methyltransferase (PRMT) family of enzymes. The study of PRMTs has gained momentum in recent years as these enzymes and their many substrates have been discovered to be pervasive and vital in numerous cellular processes (Chapter 1). Some PRMTs have also been implicated in several human diseases, further emphasizing the urgency in understanding how PRMTs function and are regulated within the cell (Chapter 1). PRMT1 and Hmt1, the predominant PRMTs in mammals and yeast (Saccharomyces cerevisiae), respectively, have been two of the main focuses in this area of study.

In order to gain further insight into how PRMTs are regulated, we have expressed and purified full-length and truncated (Δ1-146) constructs of the yeast Pop2 protein, a potential regulator of Hmt1 (Chapter 2). We have also studied its regulatory effects on several Hmt1 substrates, including histone H4 protein, and have shown Pop2 (Δ1-146) to be a concentration-dependent inhibitor of Hmt1-catalyzed methylation of this substrate (Chapter 3). We have also demonstrated that full-length Pop2 may regulate the methylation of many other substrates in vivo (Chapter 3). Additionally, we present data that supports a substrate-sequestering mechanism for methylation inhibition, and
suggest a region of Pop2 (Δ1-146) that may contribute to an important protein-protein interaction (Chapter 3). For each of these projects, a brief summary is provided below, followed by future work and directions.

**Expression and Purification of Pop2**

Pop2 was selected as a potential regulator of Hmt1 as a result of experimental evidence demonstrating that hCaf1, the human homolog of Pop2, was a regulator of PRMT1.\(^1\) hCaf1 was shown to regulate the methylation of several substrates by PRMT1, including histone H4 protein. Based on these data, we hypothesized that Pop2, like hCaf1, would regulate Hmt1-catalyzed methylation of histone H4 protein, and possibly other substrates. This chapter focused on the expression and purification of various constructs of Pop2. Although the original full-length His-tagged Pop2 showed inhibition of histone H4 protein methylation by Hmt1, its expression was rather low, and purity was in need of improvement.

The final and most successful construct of Pop2 was a truncated (Δ1-146) construct with a TEV-cleavable His-tag. Expression and overall purity of this construct both greatly increased. This specific truncation was created for two primary reasons. First, it is the same construct used by the group that solved the crystal structure of Pop2, and includes the RNase domain of Pop2.\(^2\) This allowed us to use conserved structural characteristics in exploring the regulatory mechanism of Pop2 (Chapter 3). Second, this portion of the Pop2 protein is the section of Pop2 that is conserved with human hCaf1 and *S. pombe* Pop2 sequences.\(^2,3\) This suggested that this truncation of Pop2 included the relevant part of the protein that is responsible for the function of regulating methylation.
Despite the advantages of the truncated (Δ1-146) construct of Pop2, several steps during the purification process proved problematic. The construct was exceptionally susceptible to protein precipitation during and after TEV-cleaving, which resulted in loss of most or all of the Pop2 (Δ1-146) protein during purification. However, by raising the temperature from 4°C to 16°C during these steps, the precipitation issues were immediately resolved. We concluded that this truncated (Δ1-146) construct of the Pop2 protein becomes temperature sensitive following TEV-cleavage of the His-tag. Once this hurdle was overcome, the Pop2 (Δ1-146) construct became stable in solution and could be used for further experimentation.

Regulation of Hmt1-Catalyzed Methylation by Pop2

In order to examine the effect of Pop2 on the methylation by Hmt1, activity assays were first performed with histone H4 protein substrate with [3H]-S-adenosyl-L-methionine (SAM) as a tracer. Addition of Pop2 (Δ1-146) resulted in decreased rates of methylation in a concentration-dependent manner. We concluded that Pop2 (Δ1-146) mimics hCaf1 in inhibiting the methylation of histone H4 protein, confirming this homologous function. Interestingly, there was no inhibition observed in the methylation of two other Hmt1 substrates, Npl3 and R3 peptide, in the presence of full-length Pop2.

To confirm that histone H4 protein methylation was in fact being inhibited by Pop2, S. pombe Pop2 was also added to methylation reactions with histone H4 protein. The inhibitory effect was comparable to that observed with Pop2 from S. cerevisiae. This suggested that the regulatory function of Pop2 is conserved among yeast species as well as being conserved in humans via hCaf1.

To explore the scope of Pop2 and its effects on the methylation of other substrates, several in vivo and in vitro experiments were conducted utilizing different
yeast strains. Using [$^3$H]-SAM and western blot analysis, results showed altered patterns of methylation in the presence and absence of Pop2. The first experiment involved hypomethylated yeast cell lysate from ΔHmt1 cells in reactions with exogenous Hmt1 in the presence and absence of additional full-length Pop2. The results demonstrated an overall decrease in methylation of substrates after 20 minutes in the presence of additional full-length Pop2. The second experiment examined the methylation differences in wild type and ΔPop2 yeast cells in vivo. Western blot analysis of cell lysates from these growths showed an overall increase in arginine methylation (both ADMA and MMA) from the ΔPop2 yeast cells when compared to wild type, suggesting that the loss of Pop2 allowed methylation of substrates that otherwise would not occur, or would usually occur at a lower rate.

Discovering the mechanism that Pop2 employs to accomplish the observed regulation of arginine methylation was the goal of the next set of experiments. Previous studies have shown that hCaf1 and PRMT1 colocalize in vivo, and pull-downs from yeast cell lysates have shown an interaction between Hmt1 and Pop2. Based on these data, we hypothesized that Pop2 could be directly interacting with Hmt1, preventing it from methylating its substrates. However, several pull-down, cross-linking, and immunoprecipitation experiments have yielded no evidence of such an interaction in vitro.

Finally, a pull-down using only purified histone H4 protein and Pop2 (Δ1-146) showed a direct interaction, suggesting that Pop2 inhibits methylation by the sequestration of certain substrates. This type of mechanism agrees with the apparent lack of methylation inhibition by Pop2 of the other substrates tested. Despite previous studies that have alluded to a direct interaction between PRMTs and Pop2/hCaf1, it appears that the two proteins may not directly interact at all. The novel interaction
between Pop2 (Δ1-146) and histone H4 protein suggests that there could be one or more bridging molecules that result in associations of PRMTs with Pop2/hCaf1, and possibly a three-way complex between PRMT, substrate, and Pop2.

To further explore how Pop2 inhibits methylation, we employed site directed mutagenesis on a carefully targeted surface of Pop2 (Δ1-146) that we hypothesized was involved in its interaction with histone H4 protein. Based on the crystal structure of Pop2, we chose three residues located on an alpha helix that pointed out into solution, and were on a surface that was not currently known to interact with other proteins. We mutated E260, E263, and K267 to alanines, and performed methylation activity assays with them along with wild type Pop2 (Δ1-146). Although E260A and E263A showed similar results to wild type Pop2 (Δ1-146), the K267A variant demonstrated a decreased ability to inhibit the methylation of histone H4 protein by Hmt1. This suggested that K267 and possibly a region proximal to it is involved in an important interaction responsible for the regulation of arginine methylation.

**Summary and Future Work**

This thesis has contributed to the foundation of knowledge concerning the regulation of PRMTs, an area that is currently being investigated but remains poorly understood in this field. With the implication of PRMTs and dysregulated arginine methylation in various human disease states, selective regulation of PRMTs is becoming an appealing target for pharmaceuticals. However, this can be more challenging than it seems when inhibiting one PRMT can have many widespread consequences in addition to the one targeted.

Here, we have shown selective methylation inhibition of a substrate via a novel protein-protein interaction that leaves some substrates unaffected. Further experiments
are currently needed to fully understand the effects of Pop2 on arginine methylation. However, progress is currently bottle-necked by inconsistent activity of recombinant Hmt1 from various purifications. Once Hmt1 activity is stabilized, additional experiments and methylation rate data can expound upon and define additional details of how Pop2 inhibits arginine methylation.

Future experiments could include optimizing the immunoprecipitation and cross-linking experiments described below that were unable to yield any conclusive results. Using various antibodies specific to histone H4, Hmt1, and Pop2 in such immunoprecipitation experiments could yield some exciting results as each is used in turn to pull down the other proteins. Additional work could also include similar pull-downs as well as methylation activity assays using histone H4 protein that is monomethylated at arginine 3. This would explore the questions of whether Pop2 is able to differentiate between and associate with this substrate compared to unmodified histone H4 protein, and whether the ability of Pop2 to regulate methylation is altered under these circumstances.

Various mutations and truncations of Pop2 could be created and used to further define and clarify the regions of Pop2 that are responsible for its inhibitory function. Methylation activity assays with various constructs could reveal and narrow down the smallest region of Pop2 required for methylation inhibition. Perhaps a stable complex between a construct of Pop2 and its interacting partner(s) could be isolated and purified for crystal structure determination. This would shed a great deal of light on the precise regions of Pop2 that are important for its interactions, as well as discovering the regions of histone H4 and Hmt1 that could also be involved.

Additionally, there are other substrates of Hmt1 besides histone H4 that could be influenced by interaction with Pop2. Methylation activity assays with proteins that are
predicted to interact with Pop2 could identify which of these substrates are regulated in a similar manner to histone H4. These proteins could also be used in pull-down experiments as described above to further define the mechanism employed by Pop2. All of these experiments could be done using the Pop2 protein from *S. pombe* in confirming these effects of Pop2 in another yeast species, and suggesting an evolutionarily conserved novel function for this protein.

Future work will explore the details of how specific regulation of arginine methylation is accomplished, and exploit that information in designing pharmaceuticals that can specifically target and modulate dysregulated PRMT activity without disturbing the essential roles that PRMTs play in cellular physiology. Future research will continue to uncover various ways that PRMTs are regulated, and the mechanisms of how their activity can be modified to treat and even cure human diseases.

**Additional Experiments**

Several experiments were performed when attempting to detect complexes between histone H4 protein, Pop2 (Δ1-146), and Hmt1. However, due to technical obstacles in optimizing the protocols, these experiments were inconclusive. For the sake of completeness and thoroughness, the protocols for those experiments are included below.

**Cross-Linking Experiments** – Equimolar amounts (0.3 or 0.5 μM) of histone H4 protein, Hmt1, and Pop2 (Δ1-146) were incubated together in 100 μL of PBS pH 7.5 for approximately 20 minutes. Gluteraldehyde (Sigma) was added at final concentrations of 0.025% or 0.1%, and the samples incubated at room temperature for 5 minutes. The cross-linking reactions were quenched with 10 μL of 1 M Tris-HCl pH 8.0. Samples were resolved by SDS-PAGE, and then transferred to a PVDF membrane. The western
membrane was then stained with Ponceau S Solution (Sigma) to visualize all proteins, followed by rinsing to destain. Western blots were performed on the membrane using antibodies specific to each protein: Anti-CNOT7 polyclonal antibody to detect Pop2 (Pierce, PA5-31139), anti-histone H4 (H-97) (Santa Cruz sc-10810), and anti-Hmt1 (generously provided by Dr. Michael C. Yu, Department of Biological Sciences, University at Buffalo, New York). Complexes corresponding to the correct molecular weights were not detected (Figure 4-1). However, with optimization of this protocol, viable results may be possible.

Figure 4-1. Western blots of cross-linking Hmt1, H4 protein, and Pop2 (Δ1-146). Lanes 1-5: Anti-His western blot detecting His-tagged Hmt1. 1 = Hmt1 only. 2 = Hmt1 and H4. 3 = Hmt1 and Pop2. 4 = Hmt1, Pop2, and H4. 5 = Hmt1, Pop2, and H4 with 0.1% gluteraldehyde. Lanes 6-9: Anti-H4 western blot. 6 = H4 only. 7 = H4 and Pop2. 8 = H4 and Hmt1. 9 = H4, Pop2, and Hmt1. Lanes 10-13: Anti-CNOT7 western blot detecting Pop2. 10 = Pop2 only. 11 = Pop2 and H4. 12 = Pop2 and Hmt1. 13 = Pop2, H4, and Hmt1. All other gluteraldehyde concentrations were 0.025%. Approximate molecular weights: Hmt1 42 kDa; Pop2 33 kDa; histone H4 protein 11 kDa. Approximate molecular weights of potential complexes: Hmt1 and Pop2 75 kDa; Hmt1 and histone H4 53 kDa; Pop2 and histone H4 44 kDa; Hmt1, Pop2, histone H4 86 kDa; Hmt1 dimer 84 kDa; Hmt1 dimer and histone H4 95 kDa; Hmt1 dimer and Pop2 117 kDa; Hmt1 dimer, histone H4, and Pop2 128 kDa; Hmt1 trimer 126 kDa; Hmt1 tetramer 168 kDa.
Immunoprecipitations – Protein A sepharose (Sigma) was resuspended in buffer A (20 mM phosphate, 150 mM NaCl, pH 8.0) and allowed to swell for 30 minutes, followed by equilibration with buffer A. The sepharose was allowed to incubate overnight with anti-His antibody (Santa Cruz sc-8036) and rabbit anti-mouse secondary antibody (Open Biosystems) at 4°C with agitation. The secondary antibody was used because mouse IgG1 antibodies, such as the anti-His antibody used in this experiment, do not have a very high affinity for protein A sepharose. The secondary antibody allows more of the primary antibody to bind to the sepharose. His-tagged Hmt1 was then added to the sepharose solution (final concentration 10 μM) and allowed to incubate at 4°C with agitation for 4 hours. The sepharose was then washed twice with PBS by spinning down at 1,000xg at 4°C for 1 min each time. Histone H4 protein was then added (final concentration 6 μM) and allowed to incubate for 15 minutes, followed by the addition of TEV-cleaved Pop2 (Δ1-146) (final concentration also 6 μM). The solution then incubated 2 hours at 4°C with agitation. Following incubation, the solution was centrifuged (1,000xg, 1 min, 4°C) and again washed 3 times with PBS. Samples were eluted by adding SDS sample buffer, boiling 5 minutes, centrifuging, and pipetting off the supernatant. Samples were resolved by SDS-PAGE, and bands were visualized using SYPRO Ruby Protein Gel Stain (Sigma). Obstacles with this experiment included difficulty binding the antibodies to the sepharose, and control experiments demonstrated that histone H4 protein nonspecifically binds to Protein A Sepharose. Future work in optimizing these protocols could work around these issues and present valuable data on the potential interactions taking place between Hmt1, Pop2, and histone H4 protein.

References


