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The effect of mutations in PRMT5 on its ability to oligomerize

Rachel Scarl
John Carroll University, rscarl14@jcu.edu

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By

Rachel T. Scarl

John Carroll University

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Abstract

Atherosclerosis is a cardiovascular disease caused by a chronic inflammatory response in the cells lining blood vessels, vascular endothelial cells. In these cells, a network of interacting proteins regulates inflammation. One of these proteins, PRMT5, regulates the inflammatory response by methylating other proteins involved in inflammation. For PRMT5 to carry out its function, it must first oligomerize with itself and various other proteins. In this study, I determined whether mutations to specific PRMT5 methylation sites affect its ability to oligomerize. By expressing PRMT5 in HEK293 cells and studying the effects of adding a chemical cross-linking agent, DMS, I was able to determine whether mutations to PRMT5 had an effect on cross-linking. My results show that post-translational modifications are required at specific sites in PRMT5 in order for successful oligomerization to occur.

Introduction

Atherosclerosis is a major contributor to heart disease which is the leading cause of death in the United States (Heron, 2013). Atherosclerosis is characterized by the build-up of plaque made of fat, cholesterol, and calcium in the blood vessels (Weber and Soehnlein, 2012). This vascular disease, which can directly lead to stroke, heart attack, or death, is caused by an ongoing inflammatory response in the cells lining the blood vessels. The longer the inflammation lasts in the blood vessels, the more likely a person will develop atherosclerosis (Libby, 2012). To find ways to prevent inflammation and therefore plaque build-up, researchers are focusing attention on the cells that make up the lining of blood vessels, endothelial cells (ECs). Understanding the role of ECs in the maintenance of healthy blood vessels and their role in the development of vascular diseases could lead to treatment to prevent severe inflammation and ultimately atherosclerosis.

A variety of proteins on the surface of these ECs cooperate to produce a pro-inflammatory response. These proteins include E-selectin and vascular cell adhesion molecule 1 (VCAM1)

(Bandyopadhyay, et al., 2012). E-selectin and VCAM1 are expressed on the outside of vascular endothelial cells when the cells are exposed to an inflammatory signal. These inflammatory signals can be released when certain factors are present such as high cholesterol, diabetes, obesity, and smoking (de Maat, et al., 2002). When leukocytes, white blood cells involved in the inflammatory and other immune responses, pass by the cells expressing E-selectin and VCAM1 the leukocytes attach to these proteins. Once attached to the ECs, leukocytes release various molecules that elicit additional inflammation (Figure 1). Among other responses, inflammation causes changes in gene activity mediated by proteins called transcription factors that either repress or activate gene expression. One well studied protein, HOXA9, is a transcription factor that activates a variety of genes, including genes for VCAM1 and E-selectin (Bandyopadhyay, et al., 2012). Specifically, HOXA9 activates the E-selectin gene in ECs so that E-selectin can be expressed in these cells. Because of HOXA9's role in promoting gene expression in ECs, alteration of HOXA9 activity may change E-selectin and VCAM1 expression, leading to development of vascular defects and disease (Bandyopadhyay, et al., 2007).

Not surprisingly, given that HOXA9 regulates the expression of proteins involved in the inflammatory response, HOXA9 itself is regulated to ensure that it is not constantly turning on genes. A common mechanism used by cells to regulate the activity of proteins is by making chemical modifications to proteins. These changes, known as post-translational modifications, include addition or removal of certain chemical groups on a protein that cause the protein to become more or less active (Snider, et al., 2014). Little is known about the chemical modifications that occur on HOXA9, however, it has been shown that HOXA9 interacts specifically with the enzyme PRMT5 in ECs that are currently expressing pro-inflammatory proteins (Bandyopadhyay, et al., 2012). PRMT5 transfers methyl groups, methylation, to the amino acid arginine on target proteins, such as HOXA9 (Kanamaluru, et al., 2011). Methylation is an important regulatory step in many cellular processes including the cell cycle and DNA replication (Lee, et al., 2005).

PRMT5 is a regulator of not only inflammatory responses but also other important cellular processes such as transcriptional regulation, RNA processing, signal transduction, and DNA repair (Figure 2). Since PRMT5 itself is involved in such important processes, its activity must be regulated (Karkhanis, et al., 2011). Methylosome protein 50, MEP50, interacts with PRMT5 to regulate its activity. MEP50 may regulate PRMT5's activity either by directly binding to PRMT5 or by directing PRMT5 to specific targets that need to be methylated (Wang, et al., 2013). Recently, it was found that MEP50 binds to PRMT5 complexes in order to regulate PRMT5 activity (Figure 3). Like PRMT5, MEP50 itself can modify and methylate specific arginine residues in target proteins (Ho, et al., 2013).

With respect to PRMT5's role in atherosclerosis, studies are underway to understand how PRMT5 controls the inflammatory response in ECs lining the blood vessels. Recent experiments suggest that PRMT5 directly regulates the pro-inflammatory response in ECs by interacting with and methylating the HOXA9 protein, which in turn changes the activity of specific genes including inflammatory response genes (Bandyopadhyay, et al, 2012). For PRMT5 to methylate its targets, the protein undergoes oligomerization, the formation of a complex containing multiple PRMT5 molecules and other proteins (Rho, et al., 2001). One study has shown that when PRMT5 forms multimers, MEP50 becomes part of the complex, allowing for full PRMT5 methylation activity (Antonysamy, et al., 2012). This observation suggests that oligomerization is necessary for PRMT5 function and regulation of inflammation in ECs. If PRMT5 is mutated in a way that does not allow proper oligomerization, MEP50 cannot bind to the complex and PRMT5 function is compromised (Figure 4).

These data suggest that oligomerization of PRMT5, MEP50, and other proteins is critical for proper PRMT5 function and regulation of inflammation. Therefore, understanding the factors that contribute to successful PRMT5 oligomerization may provide insight into chronic inflammation. Previous work has shown that severe inflammation results when PRMT5 is methylated (Bandyopadhyay, et al.,

2012). Mass spectrometry analysis of PRMT5 has revealed that the protein is methylated at three amino acid residues: lysine at position 200 (K200), lysine at position 240 (K240), and arginine at position 492 (R492), (Figure 5, S. Bandyopadhyay, personal communication). I hypothesized that the addition of methyl groups to these specific amino acids plays a role in PRMT5 oligomerization and formation of the activated complex responsible for regulating the inflammatory response. To investigate this hypothesis, I examined the effect of mutations that alter the methylation of these sites in PRMT5.

Methods

PRMT5 cDNAs

PRMT5 cDNA was purchased from Origene Technologies (Rockville, MD). PRMT5 was subcloned into pcDNA3 and a Myc epitope was introduced upstream of the initiating codon by PCR. PRMT5 mutant cDNAs were created by direct site mutagenesis (S. Bandyopadhyay, personal communication).

Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM medium supplemented with 15% fetal bovine serum (FBS) at 37 °C with a CO_2 concentration of 5%. Transfections were carried out with Lipofectin 2000 according to manufacturer's protocol (Life Technologies, Inc.). Briefly, 10 µg of PRMT5 expression vectors were added to cultured cells at 80% to 90% confluence for 5 hours using OptiMEM (Life Technologies, Inc.). Cells were allowed to recover for 16 to 20 hours before harvesting. Transfection efficiency was assessed in an independent experiment using a GFP expression pcDNA3 vector. Typically, at least half of the cells were transfected (data not shown).

Immunoprecipitation Assays

Immunoprecipitation was used to detect PRMT5 expression in transfected cells. Lysates from cells expressing wild-type and mutant PRMT5 were prepared in RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and phosphatase and protease inhibitor

cocktails (Life Technologies, Inc.). Lysates were incubated overnight at 4°C with rabbit monoclonal anti-Myc antibody (Santa Cruz). Protein A/G agarose beads (Santa Cruz) were added to the lysates, and the mixtures were rotated overnight at 4°C. The A/G beads were washed four times with RIPA buffer. Washed beads containing immunoprecipitates were boiled with 2X Laemmli protein sample buffer (Sigma), and proteins were subjected to SDS-PAGE followed by Western blot analysis.

Western Blot Analysis

Expression of Myc-tagged wild-type and mutant PRMT5 proteins was confirmed by Western blotting. Briefly, protein extracts was separated on a 10% SDS-PAGE gel and blotted to a membrane. Anti-PRMT5 antibody (Millipore) was used to detect proteins.

Immunoprecipitated proteins were separated on 6%, 8%, or 10% SDS-PAGE gels and then transferred electrophoretically onto Immobilon-P membrane as previously described (Bandyopadhyay, 2007). The membranes were incubated with protein-specific primary antibodies including anti-PRMT5 and anti-Myc for 5-24 hours followed by incubation with horseradish peroxidase (HRP) – conjugated specific secondary antibodies (Millipore) for 2-3 hours. Immunocomplexes were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech).

Chemical Cross-linking of Immunoprecipitates

After PRMT5 was pulled down with A/G agarose beads, immunoprecipitated proteins were chemically cross-linked using dimethyl suberimidate (DMS, Thermo Scientific). An equal volume of DMS (2 mg/mL in 20 mM HEPES, pH 8.5) was added to each immunoprecipitate . Reactions were incubated for 2 hours at room temperature. Following incubation, 4X Laemmli buffer was added to the reactions, which were then boiled to remove beads and anti-Myc antibodies in preparation for SDS-PAGE and Western blot analysis.

Results

Expression of wild-type and mutant PRMT5 in HEK293 cells

We first wanted to determine if wild-type and mutant PRMT5 could be expressed in HEK293 cells which are commonly used for expressing a variety of different proteins. Cells were transfected with expression vectors containing Myc-tagged PRMT5 wild-type or mutant cDNAs including K200A1, K200R1, K240R1, R492A1, and R492K1 (Table 1). The Myc-tag was used to separate PRMT5 from other proteins present in the cells. Cells were harvested 18-24 hours after transfection and lysed. Following immunoprecipitation, proteins were analyzed by Western blot. Wild-type and mutant PRMT5 expression was highly variable (Figure 6). The level of expression of mutant PRMT5 was much higher than the level of expression for the wild-type PRMT5 (Figure 6, compare lanes 2-8). Therefore, we determined that wild-type and mutant PRMT5 could be successfully expressed in HEK293 cells and that there appears to be a higher expression level of the mutant protein as compared to the wild-type PRMT5.

Oligomerization of PRMT5 and mutants

Next, we wanted to determine if certain mutations in PRMT5 would affect its ability to oligomerize. The mechanism behind PRMT5 oligomerization is thought to involve amino acid methylation. Since the amino acid residue K200 in the PRMT5 protein is known to be methylated (S. Bandyopadhyay, personal communication), we wanted to determine if mutating this lysine would affect the methylation process and therefore affect PRMT5's ability to oligomerize.

To do this, HEK293 cells were transfected with wild-type and mutant PRMT5 Myc-tag expression vectors, and cells were harvested and lysed 18-24 hours after transfection. After separation of the Myc-tagged PRMT5 proteins from lysates, wild-type and mutant PRMT5 were aliquoted into two groups.

One aliquot was a non-cross-linked control and the other aliquot was treated with DMS. All samples were analyzed by Western blot with anti-Myc or anti-PRMT5 primary antibodies (Figures 7 and 8).

There is evidence of chemical cross-linking when DMS is applied to wild-type PRMT5 as opposed to when DMS is absent and no cross-linking occurs (Figures 7, compare lanes 1 and 2). Cross-linking is seen in the multiple higher molecular weight bands seen in lane 2 that are not visible in lane 1. This is evidence that PRMT5 is present in oligomers under these conditions. From this data, however, we are unable to determine whether other proteins comprise the oligomers besides PRMT5 and what the stoichiometry of these proteins is. The bands that are present below the PRMT5 band are probably PRMT5 degradation products (Figure 7, lane 1).

All PRMT5 mutants tested show evidence of oligomerization when DMS was applied, but to varying degrees. In the K240R1 mutant, a smaller amount of cross-linked protein is seen as compared to the cross-linked wild-type PRMT5 (compare lanes 2 and 4, Figure 8). There is an even smaller amount of chemically cross-linked proteins in the R492A1 mutant as compared to the wild-type PRMT5 (compare lanes 2 and 6, Figure 8). The K240R1 mutant had a smaller effect on PRMT5 oligomerization than the R492A1 mutant did. The mutant R492K1 cross-linking results show about the same amount of protein is cross-linked as compared to the wild-type PRMT5. This suggests that the mutation from an arginine to a lysine at position 492 has little or no effect on PRMT5 oligomerization (compare lanes 2 and 8, Figure 8).

From these results, I concluded that although mutations to PRMT5 methylation sites do not completely prevent oligomerization of PRMT5, some mutations such as K240R1 and R492A1 reduce the degree of PRMT5 oligomerization. However, in some cases, such as the R492K1 mutant, oligomerization is only slightly affected. This may indicate that each site examined in this study may have a distinct role in forming oligomers.

Discussion

In this project, I showed that certain mutations in PRMT5 can affect its ability to oligomerize while other mutations seem to have little effect on oligomerization. For example, the PRMT5 mutants K240R1 and R492A1 show less oligomerization than the wild-type PRMT5 (lanes 2, 4 and 6, Figure 8). On the other hand, the R492K1 mutant shows about the same amount of oligomerization as the wild-type PRMT5 (lanes 2 and 8, Figure 8). This similarity raises the possibility that lysine can substitute for arginine and that lysine can be methylated to allow PRMT5 oligomerization. However, different amino acid residues undergo distinct post-translational modifications which may account for differences seen in PRMT5 oligomerization. For example, the lysine residue normally found at position 240 in PRMT5 can be acetylated or methylated (S. Bandyopadhyay, personal communication). When changing this residue to an arginine, less PRMT5 oligomerization is seen. Even though arginine can be methylated, as can lysine, arginine can only be dimethylated while lysine can be trimethylated, and the trimethylation at this specific site may be necessary for full PRMT5 oligomerization. The fact that an arginine mutation at this site causes a lower level of PRMT5 oligomerization suggests that trimethylation at this site may be critical for full oligomerization of PRMT5.

An alanine substitution at position 492 in PRMT5 has the most detrimental effect on PRMT5 oligomerization (Figure 8, lane 6). Alanine cannot be methylated like arginine and lysine can (S. Bandyopadhyay, personal communication). Therefore, methylation at this specific site may be necessary for PRMT5 oligomerization, and without an amino acid residue that can be methylated at this site, a significant decrease in PRMT5 oligomerization is seen.

Although the data show that there is a decrease in oligomerization in mutant PRMT5 proteins possibly related to the methylation sites, experiments with more mutations to PRMT5 methylation sites need to be conducted. Such mutations should include neutral and negatively charged amino acid

residues in order to determine if positively charged amino acid residues are critical for PRMT5 oligomerization. For example, the arginine at position 492 could be changed to glutamic acid to determine the effect of a negatively charged amino acid at this site. At the same time, it would be helpful to determine the methylation status of each of the mutated residues by mass spectrometry. This may give some indication whether the residue is essential for oligomerization by methylation.

MEP50 is known to form a complex with PRMT5 during oligomerization, but we did not know if MEP50 is present in the PRMT5 oligomers. To test the effect of PRMT5 mutations on MEP50's ability to oligomerize with PRMT5, Myc-tagged PRMT5 oligomers could be isolated by immunoprecipitation, chemically cross-linked and analyzed by Western blotting with an anti-MEP50 antibody. This would reveal if mutations in PRMT5 affect MEP50's ability to oligomerize with PRMT5. Another experiment that would be helpful is to determine if the mutated PRMT5 proteins contain any catalytic activity, and if so, to compare that catalytic activity to the wild-type PRMT5 activity. By determining the mutant PRMT5's activity, the mutation that inhibits PRMT5 from regulating proteins involved in inflammation can be determined.

In conclusion, the results from this preliminary study suggest that specific amino acids are important in PRMT5 oligomer formation and potentially regulation of inflammation. Future research is needed to understand the role of specific modifications to specific amino acids in order to clarify how PRMT5 oligomerizes. Because of PRMT5's role in regulating inflammation, PRMT5 could potentially be a drug target for treating atherosclerosis and other heart diseases.

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Figure 1. Role of E-selectin and VCAM1 in vascular inflammation. E-selectin and VCAM1 recruit leukocytes to vascular endothelial cells. Binding of leukocytes to VCAM1 and E-selectins triggers release of cytokines, leading to inflammation.

Figure 2. Regulatory roles of PRMT5 (S. Bandyopadhyay, personal communication). A) PRMT5 is a cofactor for various proteins involved in regulation of gene expression. B) PRMT5 is a component of multiple protein complexes that are involved in RNA transport and splicing. C) PRMT5 influences pathways that regulate the cell cycle. D) PRMT5 is involved in DNA repair.

Figure 3. Three-dimensional structure of PRMT5-MEP50 (Ho, 2013). A) Space filling model of PRMT5 and MEP50 hetero-octamer with 4 molecules each of PRMT5 and MEP50. B) Ribbon model of PRMT5 and MEP50 hetero-octamer.

Figure 4. PRMT5 oligomer formation. A) Oligomer formation is dependent on methylation of wild-type PRMT5. B) Mutation of methylation sites renders PRMT5 unable to oligomerize and form a PRMT5-MEP50 complex.

Figure 5. Schematic diagram of locations of PRMT5 mutations examined in this study.

Figure 6. Expression of wild-type and mutant PRMT5 in HEK293 cells. HEK293 cells transfected with wild-type and mutant Myc-tagged PRMT5 expression vectors were harvested 18-24 hours after transfection. The proteins were extracted and PRMT5 was isolated by immunoprecipitation with anti-Myc antibody. Immunoprecipitates were analyzed on a 10% SDS-PAGE gel followed blotting and detection with anti-PRMT5 primary antibody. The arrow indicates the PRMT5 protein at approximately 75 kDa.

Figure 7. Analysis of chemical cross-linking of wild-type PRMT5 immunoprecipitates. HEK293 cells were transfected with wild-type Myc-tagged PRMT5 expression vectors and the cells were harvested 18-

24 hours after transfection. The proteins were extracted by immunoprecipitation with anti-Myc antibody followed by cross-linking with DMS. Proteins were analyzed using a 8% SDS-PAGE gel and immunoblotting using anti-Myc primary antibody. The arrow indicates the monomer PRMT5 at approximately 75kDa; -, no DMS; +, DMS added.

Figure 8. Analysis of chemical cross-linking of wild-type and mutant PRMT5. HEK293 cells were transfected with wild-type and mutant Myc-tagged PRMT5 expression vectors and the cells were harvested 18-24 hours after transfection. The proteins were extracted by immunoprecipitation with anti-Myc antibody followed by cross-linking with DMS. The results were analyzed using a 8% SDS-PAGE gel and immunoblotting with anti-PRMT5 primary antibody. The arrow indicate the monomers of PRMT5 at approximately 75kDa; -, no DMS; +, DMS added.

Table 1: PRMT5 Mutants Examined in this Study

Abbreviation	Position	Wild-type Amino Acid	Mutant Amino Acid
K200A1	200	Lysine (K)	Alanine (A)
K200R1	200	Lysine (K)	Arginine (R)
K240R1	240	Lysine (K)	Arginine (R)
R492A1	492	Arginine (R)	Alanine (A)
R492K1	492	Arginine (R)	Lysine (K)

Figure 1

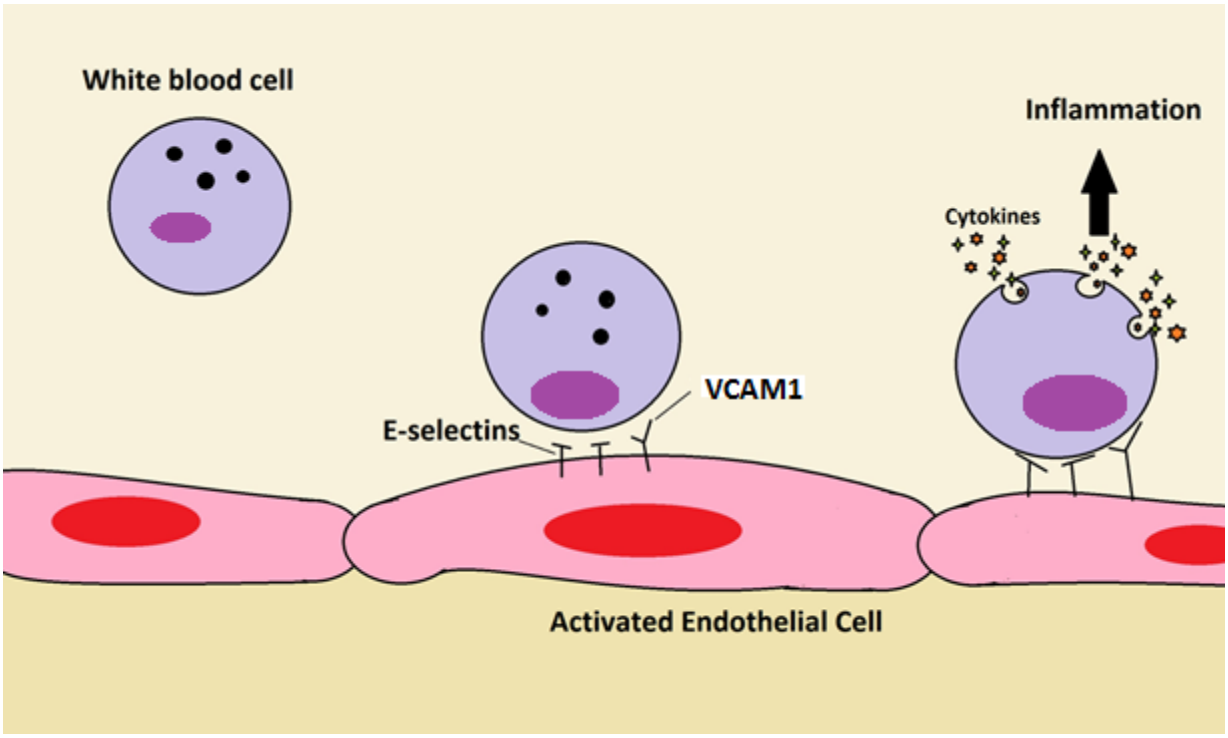

White blood cell
Inflammation
Cytokines
VCAM1
E-selectins
Activated Endothelial Cell

Figure 2

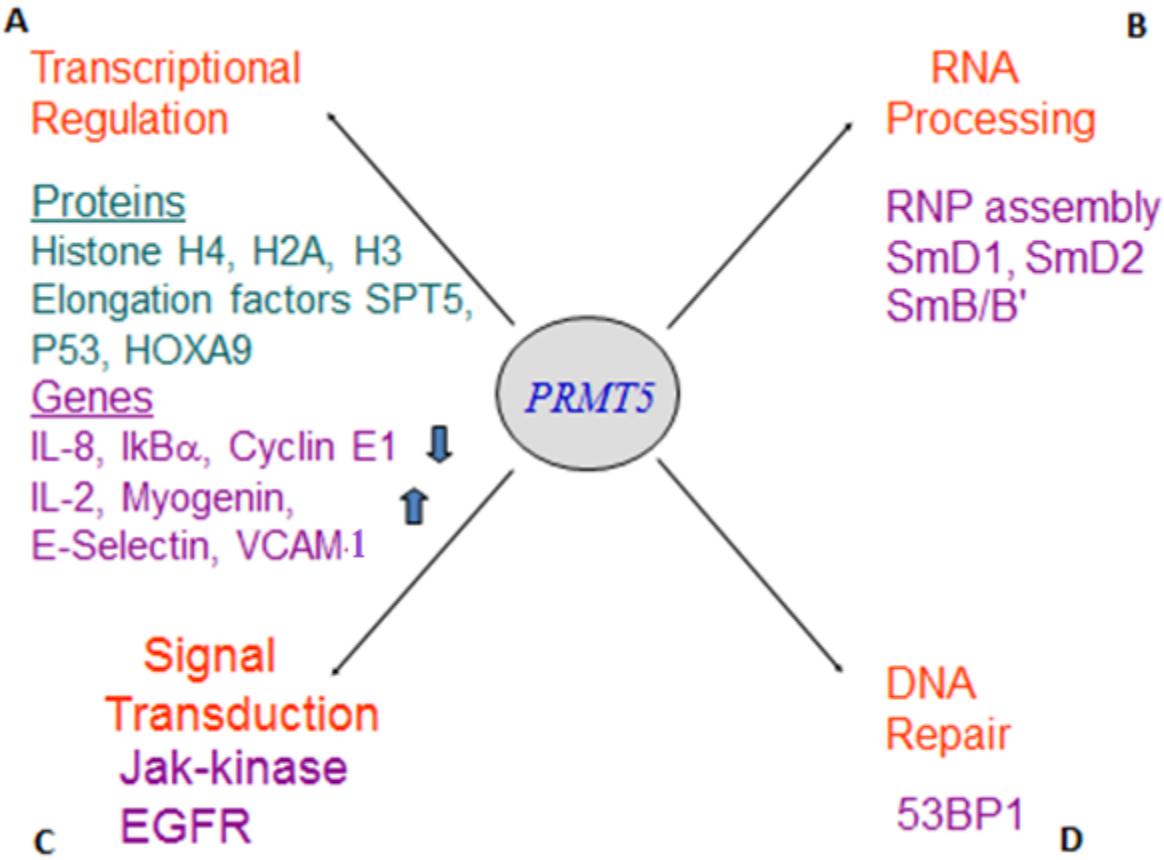
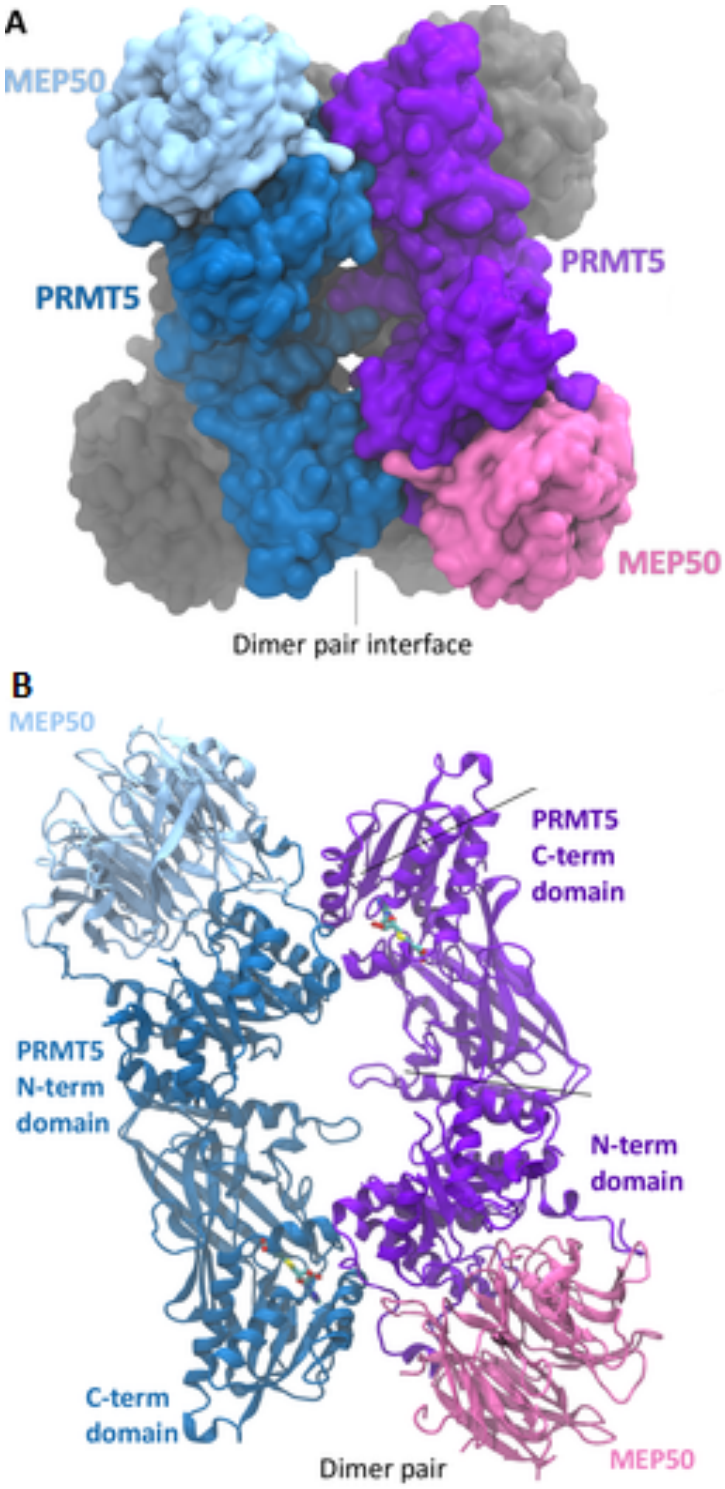

A
Transcriptional Regulation
Proteins
Histone H4, H2A, H3
Elongation factors SPT5, P53, HOXA9
Genes
IL-8, IkBα, Cyclin E1
IL-2, Myogenin, E-Selectin, VCAM-1
B
RNA Processing
RNP assembly
SmD1, SmD2
SmB/B'
PRMT5
C
Signal Transduction
Jak-kinase
EGFR
D
DNA Repair
53BP1

Figure 3


A
MEP50
PRMT5
PRMT5
MEP50
Dimer pair interface
B
MEP50
PRMT5
C-term
domain
PRMT5
N-term
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N-term
domain
C-term
domain
Dimer pair
MEP50

Figure 4

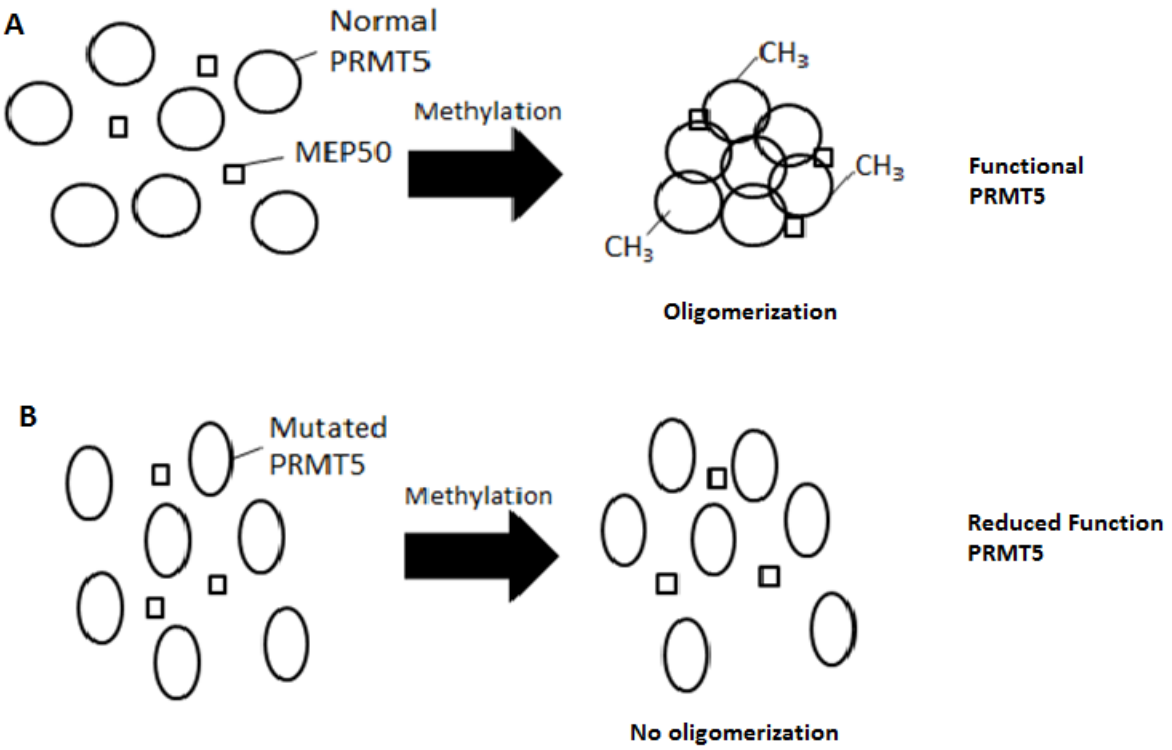
A
Normal
PRMT5
MEP50
Methylation
CH3
CH3
CH3
Functional
PRMT5
Oligomerization
B
Mutated
PRMT5
Methylation
Reduced Function
PRMT5
No oligomerization

Figure 5

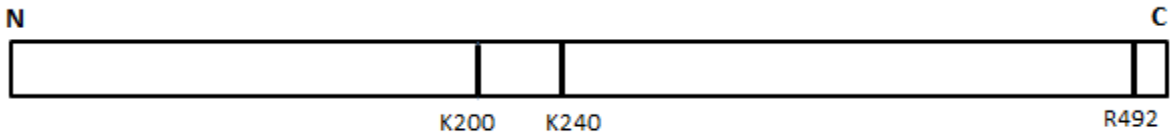

Figure 6

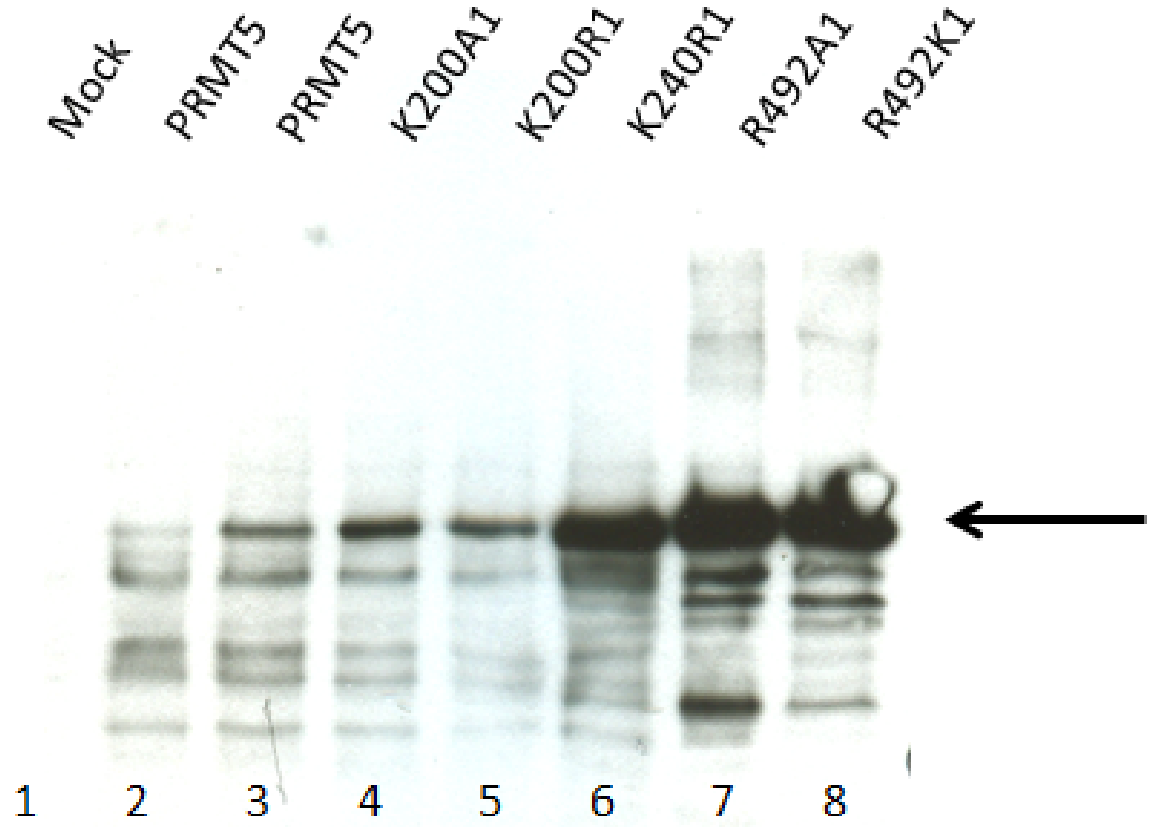
Mock
PRMT5
PRMT5
K200A1
K200R1
K240R1
R492A1
R492K1
1
2
3
4
5
6
7
8

Figure 7

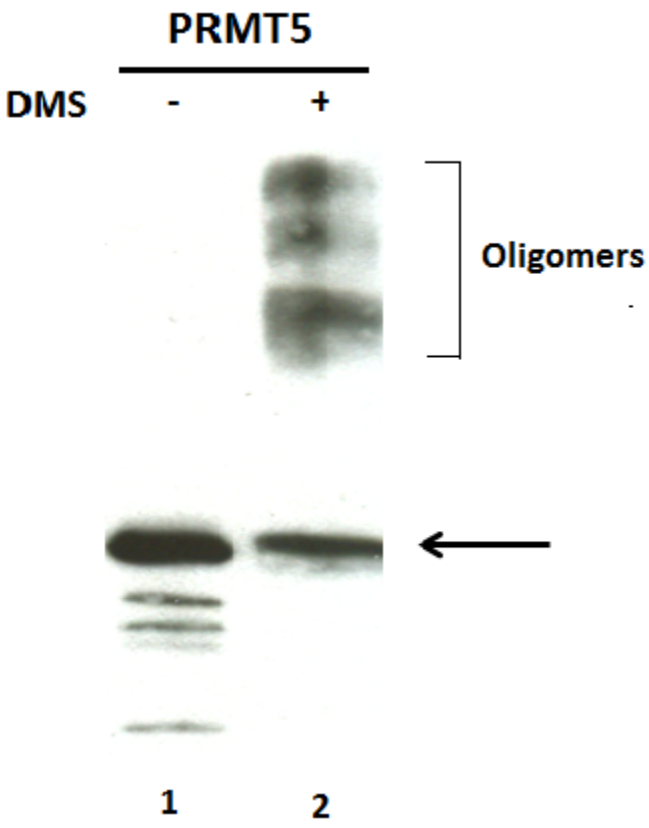

Figure 8