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ICBP90 Regulation of DNA Methylation, Histone Ubiquitination, and Tumor Suppressor Gene Expression in Breast Cancer Cells

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14. ABSTRACT
In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation. This research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1 and the human SRA-RING protein UHRF1. Additionally, it will closely examine the methylcytosine-binding specificity of UHRF1, with a specific focus on non-CpG contexts. The proposed work is ongoing, and so far the major accomplishments include creation of relevant plant lines and development of in vitro assays.

15. SUBJECT TERMS
DNA methylation, UHRF1, VIM1, ubiquitination, epigenetics, chromatin

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Table of Contents

	Page
Introduction…………………………………………………………..……..…..	4
Body……………………………………………………………………………...	4
Key Research Accomplishments………………………………………..……..	8
Reportable Outcomes………………………………………………………………	8
Conclusion…………………………………………………………………………	8
References………………………………………………………………………….	9

INTRODUCTION

DNA methylation is an important regulator of genome function, and disruptions in DNA methylation play a role in many types of cancers. Several studies have identified specific DNA methylation patterns as prognostic markers for breast cancer[1-4]. In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation[5,6]. These proteins also regulate cellular processes relevant to breast cancer pathology, including gene expression and the cell cycle[7-10]. Each protein in the SRA-RING family contains a methylcytosine-binding SRA (SET- and RING-associated) domain, a PHD domain, and one or more RING domains. Studies in mammalian cell lines have shown that the RING domain of ICBP90/UHRF1, a SRA-RING protein that is often misregulated in cancers, can target core histones or DNMT1 for ubiquitination[11,12]. Although these studies have provided valuable insight into the function of UHRF1 in epigenetic regulation and heterochromatin structure, they have not exhaustively considered other potential substrates for UHRF1 ubiquitin ligase activity. A significant portion of my research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1, a UHRF1 ortholog with a similar domain structure and comparable roles in epigenetic regulation (Fig 1). I will follow up on these studies with in vitro experiments involving UHRF1 aimed at further understanding the substrates and specificities of its RING and SRA domains. This research will provide details on fundamental epigenetic mechanisms that are central to the molecular pathology of breast cancer.

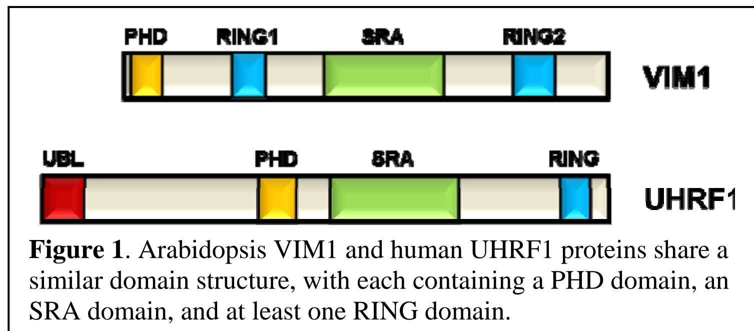

Figure 1. Arabidopsis VIM1 and human UHRF1 proteins share a similar domain structure, with each containing a PHD domain, an SRA domain, and at least one RING domain.

BODY

Here, I discuss training and research accomplishments associated with a revised Statement of Work (SOW) submitted as Amendment P00004 to project W81XWH-10-1-0080, effective August 11, 2011.

Training Plan

Task 1: Complete coursework that will provide a background in the molecular biology of breast cancer

1. "Regulation of Cell Proliferation, Senescence, and Death," which covers topics in the cell cycle and signal transduction, including lectures on oncogenesis (Months 2 – 5)
2. "Cellular and Molecular Pharmacology," which surveys receptor mechanisms and signaling pathways, including topics in drug-receptor interactions, gene expression, and chemotherapy (Months 9 – 12)

My graduate coursework has included "The Molecular Basis of Human Disease," which covered several weeks' worth of material specifically related to the molecular and epigenetic basis of cancer; "Epigenetics," which provided an understanding of basic epigenetic mechanisms as well as their significance in cancer and disease; "Genomes as Chromosomes," a minicourse that enhanced my understanding of genome function and also covered cancer-related topics; and "The Nucleus," which covered chromatin structure, transcription, RNA processing, and other nuclear processes important for understanding the molecular basis of cancer. In addition, I have taken many courses that provide a basic knowledge of biochemistry, molecular biology, and genetics that is essential for a successful research career. My coursework has gone a different direction than I anticipated when writing my SOW, and I have not taken either of the two courses that I proposed. However, I am confident that the coursework I have completed provides me with a strong academic background for breast cancer research.

Task 2: Regularly attend meetings with Cornell laboratories that study topics related to breast cancer and epigenetics

1. Monthly journal club meetings at the Center for Vertebrate Genomics (CVG) which discusses papers on cancer and is attended by members of the Nikitn and Weiss labs, which study molecular processes involved in cancer pathogenesis (Monthly, Months 1-36)
2. Monthly meetings with the Cornell Epigenetics and Chromatin Collective (EpiC), which is attended by numerous epigenetics researchers on campus and regularly features presentations on breast cancer-related topics

Since I have transitioned to a non-vertebrate model system, I have not been attending the monthly CVG meetings. I had been regularly attending the EpiC monthly meetings, but as of 2011 this group is no longer active on the Cornell campus. I continually watch for new opportunities to attend meetings with other Cornell University cancer researchers, and I attend as many breast cancer-related seminars as possible.

Task 3: Interact with Cornell University breast cancer researchers at on-campus events

1. Present at the annual CVG symposium, where I will interact with members of the CVG and discuss my research with other scientists in the field of cancer research (Yearly, Months 1 – 36)
2. Attend the bi-annual Cancer and Environment Forum held by the Breast Cancer and Environmental Risk Factors program (Twice yearly, Months 1 – 36)
3. Regularly discuss research progress with Scott Coonrod, who is a co-mentor of the research project

Since my transition to Arabidopsis as a model system, I have not recently been active with the Center for Vertebrate Genomics. The BCERF program is also no longer active at Cornell. However, I continue to meet regularly with Scott Coonrod, who is a co-mentor of my research project and a member of my thesis committee. Dr. Coonrod is a fellow epigenetics researcher who has offered great insight in our discussions, and I look forward to his continuing support.

Task 4: Present at national conferences on epigenetics and breast cancer (Yearly, Months 1 – 36)

1. The American Association for Cancer Research annual meeting
2. DOD Breast Cancer Research Program's Era of Hope meeting
3. FASEB Summer Research Conferences

Breast cancer and epigenetics-related conferences I have attended include: 1) The FASEB Summer Research Conference on Biological Methylation: From DNA to Histones, June 6-11, 2010 in Carefree, Arizona; 2) The DOD Breast Cancer Research Program's Era of Hope meeting, August 2-5, 2011 in Orlando, Florida; and 3) The Keystone Joint Symposium on Chromatin Dynamics and Epigenomics, January 17-22, 2012, in Keystone, Colorado. At each of these meetings, I have given poster presentations and had valuable exchanges with cancer and epigenetics researchers.

Research Plan

Task 1: Investigate the role of the VIM1 RING domain in epigenetic regulation in *Arabidopsis thaliana*

1. Identify substrates for VIM1 E3 ubiquitin ligase activity using a comparative proteomics approach in wild-type plants and RING domain mutants (Months 1 - 18)

To understand the contributions of individual VIM1 domains in epigenetic regulation,I have transformed *vim1 -/-* mutants with T-DNA constructs for expression of the *VIM1* gene under its native promoter. Through site-directed mutagenesis, point mutations have been made in zinc-chelating residues of one or both of the VIM1 RING domains in these expression constructs. Thus, the only form of VIM1 expressed in these plant lines will be a version containing point mutations that should abolish RING domain activity. Additionally, since the function of the VIM1 PHD domain is also poorly understood, I have created a PHD point mutant line for further study (Fig. 2).

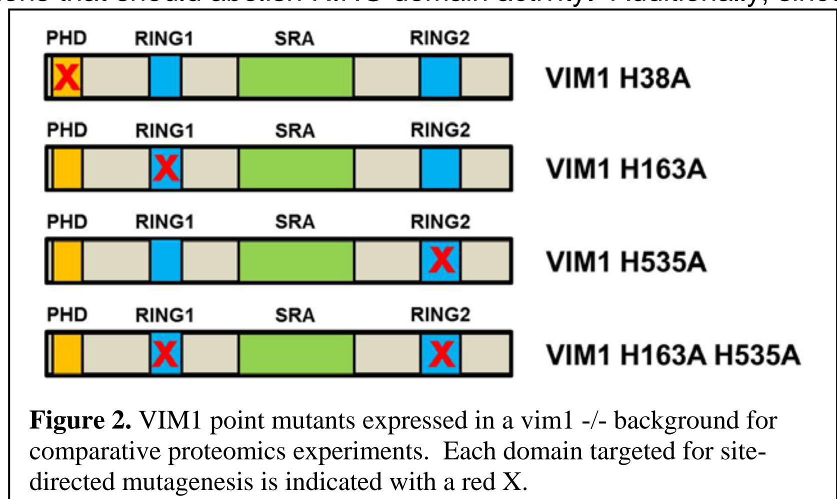

Figure 2. VIM1 point mutants expressed in a vim1 -/- background for comparative proteomics experiments. Each domain targeted for site-directed mutagenesis is indicated with a red X.

I intend to use these plant lines in future studies that compare protein levels between wild type plants and the VIM1 RING domain mutants using iTRAQ (isobaric tag for relative and absolute quantitation). In this experiment, nuclear protein from wild-type plants and *vim1 -/-* plants expressing the *VIM1* RING mutant constructs will be digested and labeled with different chemical tags. The samples will then be pooled and analyzed via nano

liquid chromatography followed by tandem mass spectrometry. The two tags will generate distinct reporter ions when fragmented, allowing for relative quantitation of proteins between the samples. Significant abundance of a protein in the RING mutant plants relative to wild type may suggest that VIM1 participates in turnover of that protein, consistent with the fact that ubiquitination often targets proteins for proteasome-mediated degradation.

2. Confirm ubiquitination targets using an in vitro assay containing purified recombinant VIM1 (Months 1 – 36)

Though I have not yet completed the proteomic analysis described above, I have performed additional biochemical analyses that provide insight on the functional importance of the VIM1 PHD domain and suggest that histones are possible VIM1 ubiquitination targets. PHD domains often act as "readers" of epigenetic marks through specific interactions with modified histones. Consequently, I used commercially available modified histone peptide arrays to screen for binding interactions between the VIM1 PHD domain and a wide variety of histone marks. These experiments have identified methyl marks on histone H3 lysine 27 as as binding targets for the VIM1 PHD domain, suggesting that VIM1 may recognize and colocalize with these marks in the genome (Fig. 3). The PHD domain also interacts with histones H4 and H2B, though these interactions appear to be less specific to certain modifications. Since VIM1 directly interacts with three of the four core histones via its PHD domain in -vitro, histones are plausible targets for ubiquitination by its RING domains.

I have developed an in vitro ubiquitination system that will be useful for assaying the ubiquitination of histones and other candidate substrates. In these assays, the required E1, E2, and E3 enzymes are combined in vitro under appropriate buffer conditions with other necessary components

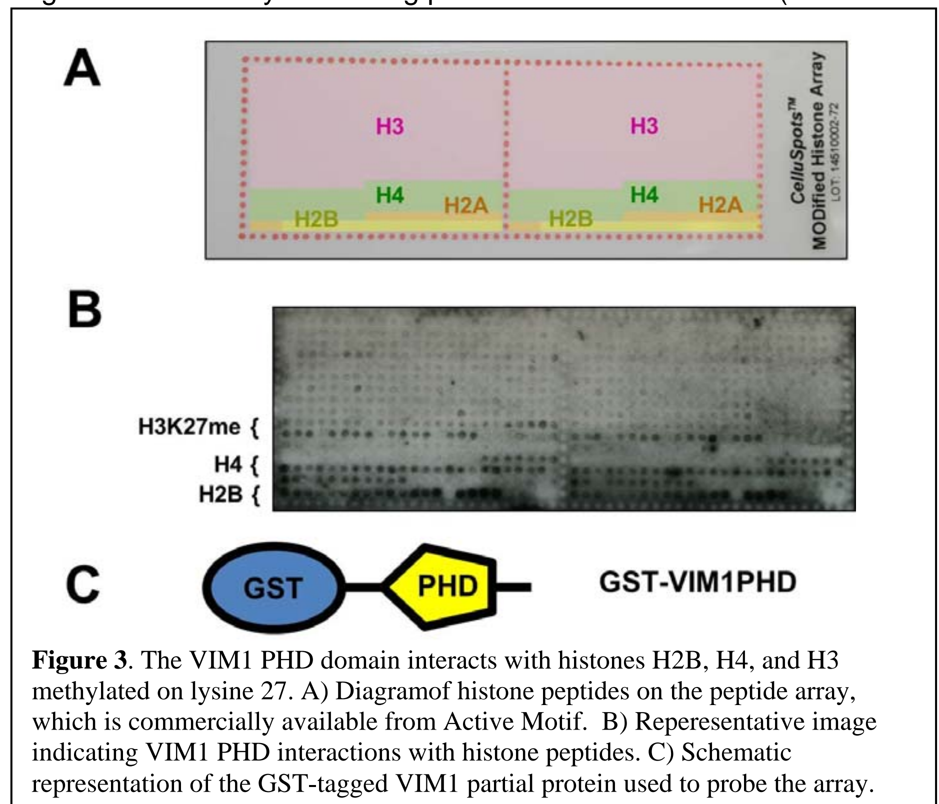

Figure 3. The VIM1 PHD domain interacts with histones H2B, H4, and H3 methylated on lysine 27. A) Diagramof histone peptides on the peptide array, which is commercially available from Active Motif. B) Reperesentative image indicating VIM1 PHD interactions with histone peptides. C) Schematic representation of the GST-tagged VIM1 partial protein used to probe the array.

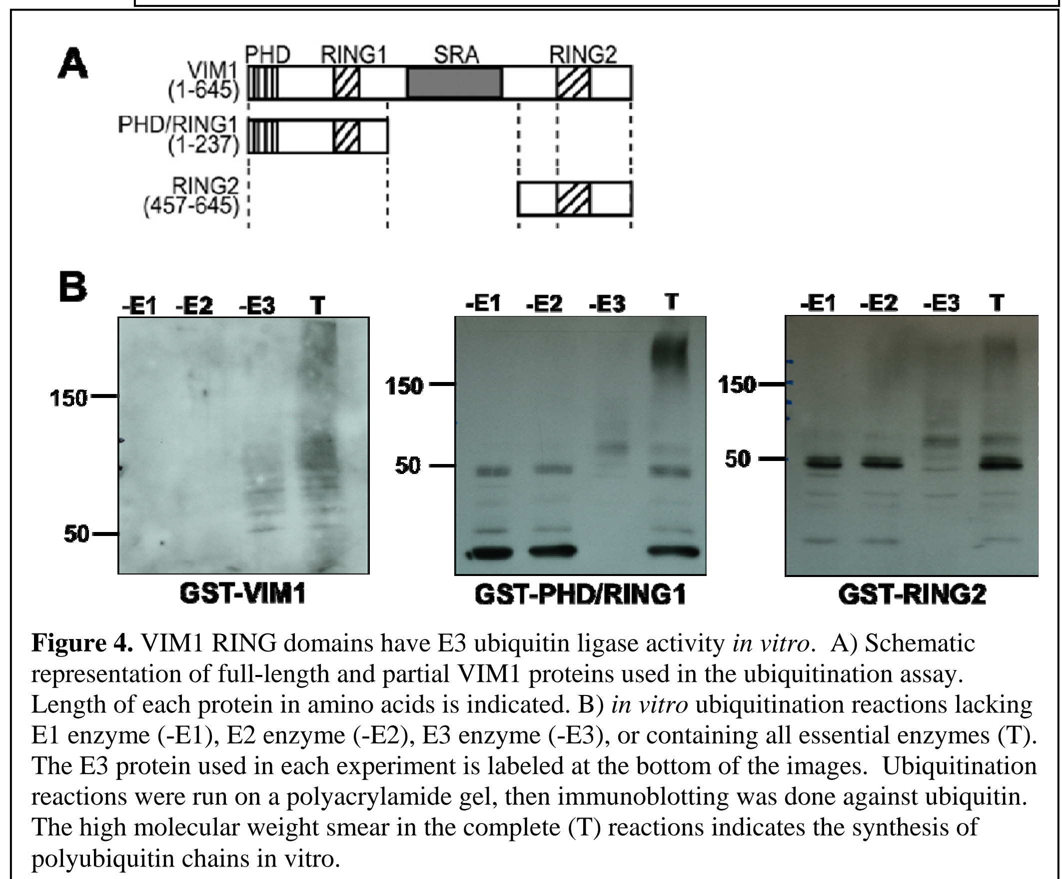

Figure 4. VIM1 RING domains have E3 ubiquitin ligase activity *in vitro*. A) Schematic representation of full-length and partial VIM1 proteins used in the ubiquitination assay. Length of each protein in amino acids is indicated. B) *in vitro* ubiquitination reactions lacking E1 enzyme (-E1), E2 enzyme (-E2), E3 enzyme (-E3), or containing all essential enzymes (T). The E3 protein used in each experiment is labeled at the bottom of the images. Ubiquitination reactions were run on a polyacrylamide gel, then immunoblotting was done against ubiquitin. The high molecular weight smear in the complete (T) reactions indicates the synthesis of polyubiquitin chains in vitro.

required for ubiquitination, including ubiquitin and ATP. Commercially available yeast UBE1, purified recombinant GST-UBC8, and purified recombinant GST-VIM1 act as the E1, E2, and E3 respectively. Using this system, I have been able to recapitulate results previously reported by another laboratory, demonstrating that VIM1 has E3 ubiquitin ligase activity in vitro[13]. In addition, I have used partial recombinant fragments of VIM1 to show that each of the two individual RING domains has activity (Fig. 4).

3. Assess the effects of RING domain mutations on DNA methylation and heterochromatin structure in vivo (Months 1 - 36)

I am using the plant lines expressing the point mutants described in Task 1, section 1 to determine the relationship between VIM1 ubiquitin ligase activity and other epigenetic processes, such as maintenance of DNA methylation and heterochromatin structure. I am currently working with a summer undergraduate intern, Elena Cravens, to examine the DNA methylation status of centromeric repeat sequences in these lines. Specifically, we are digesting genomic DNA extracted from the plant lines with HpaII, a methylation-senstive enzyme, and then visualizing the digestion pattern using southern blots against a 180-base pair centromere repeat. While fully methylated repeats appear undigested in this assay, regular cutting of unmethylated repeats yields a ladder-like digestion pattern in hypomethylated samples. As a control, we also digest each sample with MspI, an isoschizomer that is not sensitive to CpG methylation. To date, our results suggest that the RING domains have distinct functions in relation to maintenance of DNA methylation (Figure 5). The C-terminal RING domain appears to be required for maintaining methylation of the repeats, while the N-terminal RING domain is not. We are in the process of confirming these preliminary results through further experiments. In addition, we are confirming transgene expression in each line using qRT-PCR and western blotting.

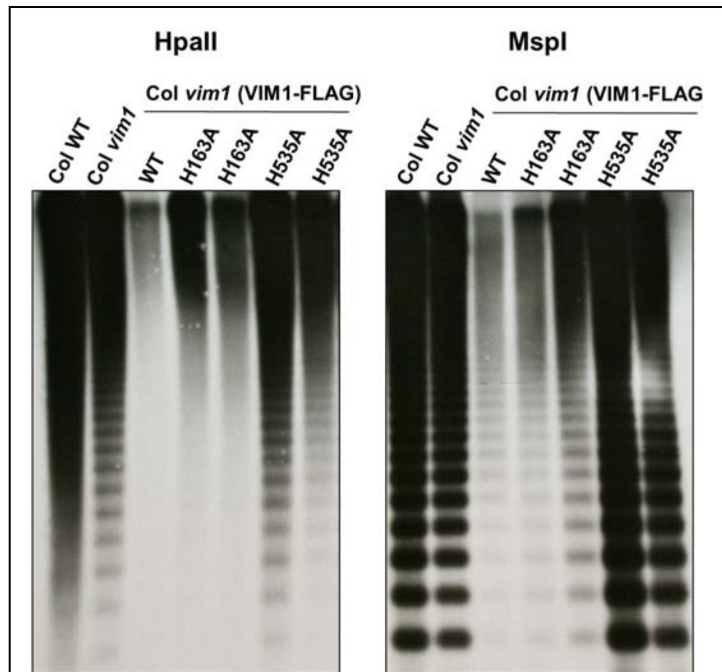

Figure 5. The VIM1 N- and C-terminal RING domains function differently in the maintenance of DNA methylation at centromere repeats. Genomic DNA was digested with HpaII or MspI as indicated, then probed in southern blots with a 180-bp centromere repeat probe. Col WT and Col *vim1* are shown as methylated and hypomethylated controls, respectively. The remaining lanes show samples from different Col *vim1* individuals expressing WT or point mutant VIM1-FLAG.

Other chromatin marks, such as histone modifications, will also be compared between wild type and mutant plants via chromatin immunoprecipitation (ChIP). Additionally, differences in centromere structure between wild-type and VIM1 RING domain mutants will be examined via cytological techniques such as fluorescence in situ hybridization (FISH) against centromeric repeats. Previously, the Richards laboratory and colleagues reported that centromere structure is significantly disrupted in *vim1 -/-* mutants, but it is unknown whether loss of RING domain activity plays a role in this phenotype[6].

Task 2: Determine the *in vitro* specificities of the human UHRF1 RING and SRA domains

1. Examine UHRF1 E3 ligase activity on human homologs of VIM1 substrates in vitro (Months 18 – 36)

These experiments have not been initiated.

2. Determine the methylcytosine-binding activity of the UHRF1 SRA domain using electrophoretic mobility shift assays (EMSA) (Months 1-24)

The purpose of this work is to closely examine the mechanism of methylcytosine binding in vitro, with special attention given to sequence context. Although it has been previously reported that the mouse homolog of UHRF1 specifically recognizes methylcytosines in CpG contexts[5], I intend to determine whether the human protein has the same specificity. The prospect of UHRF1 binding methylcytosine in non-CpG contexts is particularly intriguing, since the existence of non-CpG methylation has recently been reported in humans[14,15]. I have been working on these experiments together with Erika Hughes, a research technician in the Richards lab. Together, we have cloned and purified several UHRF1 partial proteins containing the SRA domain, and created several methylated double-stranded oligonucleotides for use as substrates (Fig. 6). We are currently optimizing the EMSA protocol using a commercial digoxigenin labeling and detection kit.

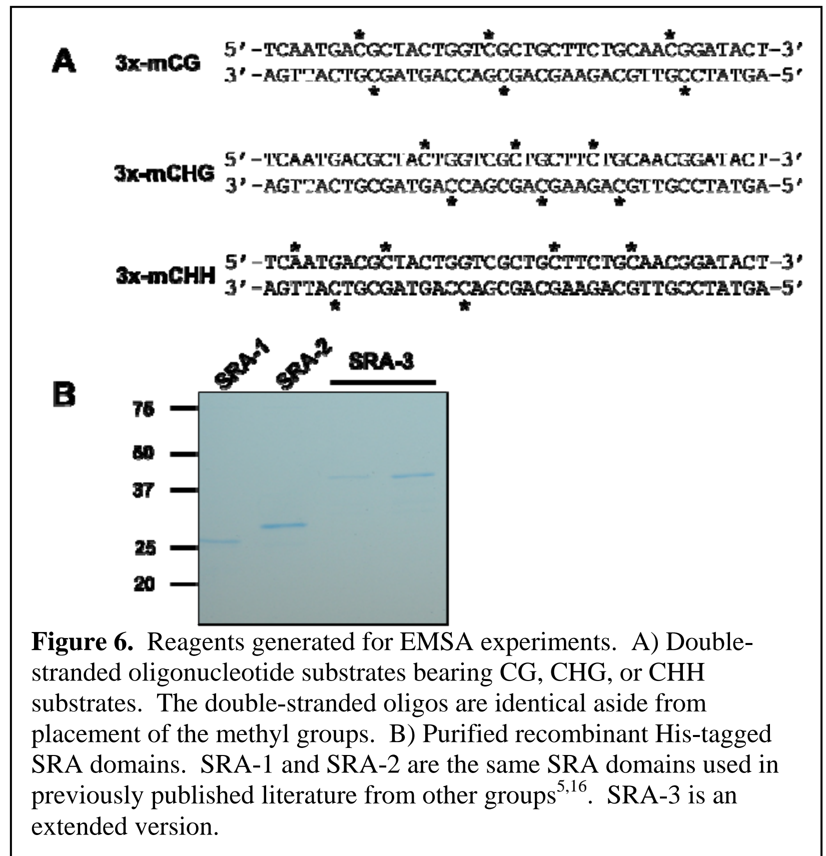

Figure 6. Reagents generated for EMSA experiments. A) Double-stranded oligonucleotide substrates bearing CG, CHG, or CHH substrates. The double-stranded oligos are identical aside from placement of the methyl groups. B) Purified recombinant His-tagged SRA domains. SRA-1 and SRA-2 are the same SRA domains used in previously published literature from other groups[5,16]. SRA-3 is an extended version.

KEY RESEARCH ACCOMPLISHMENTS

- Creation of plant lines expressing VIM1 RING point mutants under a native promoter in a *vim1* -/- background. These will be useful for two of the experiments discussed in Task 1.
- Discovery of direct interactions between the VIM1 PHD domain and specific histones, including H2B, H4, and H3 with lysine 27 methylation
- Development of an in vitro ubiquitination assay for confirmation of candidate VIM1 substrates
- Confirmation of VIM1 E3 ubiquitin ligase activity in vitro, and demonstration that each of the two VIM1 RING domains independently has activity
- Preliminary finding that the VIM1 N-terminal and C-terminal RING domains have distinct roles in maintenance of DNA methylation
- Purification of UHRF1 SRA domains and generation of oligonucleotide substrates for EMSA experiments

REPORTABLE OUTCOMES

- Poster presentation, “In vitro mechanism of UHRF1 methylcytosine binding,” at Era of Hope Conference, August 5 2011
- Poster presentation, “Binding specificity of the Arabidopsis VIM1 PHD domain,” at Keystone Symposium on Chromatin Dynamics, January 20, 2012

CONCLUSION

Though most of the experiments in this project are still ongoing, they have potential to provide information on fundamental epigenetic mechanisms. In particular, this work will uncover new substrates for the ubiquitin ligase activity of SRA-RING proteins, and further elucidate the specificities of their SRA domains. Understanding how these proteins function on the molecular level can eventually lead to the development of new epigenetically based breast cancer therapies and diagnostic tools. Short-term efforts will focus on the proposed proteomics experiments for identification of new ubiquitinated substrates, confirmation of these substrates using in vitro assays, and optimization of the EMSA protocol.

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