

AWARD NUMBER: W81XWH-10-1-0080

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

PRINCIPAL INVESTIGATOR: Molly Shook

CONTRACTING ORGANIZATION: Cornell University
Ithaca, NY 14850

REPORT DATE: July 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 July 2011		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Jul 2010 – 30 Jun 2011	
4. TITLE AND SUBTITLE ICBP90 Regulation of DNA Methylation, Histone Ubiquitination, and Tumor Suppressor Gene Expression in Breast Cancer Cells				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0080	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Molly Shook E-Mail: mss338@cornell.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cornell University Ithaca, NY 14850				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
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, ubiquitination, epigenetics, chromatin					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusion.....	6
References.....	7

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 cancer¹⁻⁴. 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 cycle⁷⁻¹⁰. 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.

BODY

Here, I discuss research accomplishments associated with a revised Statement of Work (SOW) recently submitted to the Cornell University Office of Sponsored programs. DOD approval of this SOW is pending.

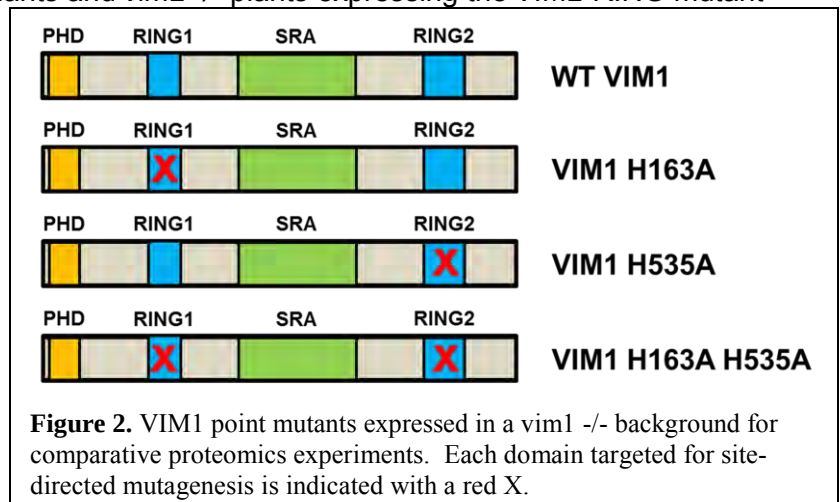
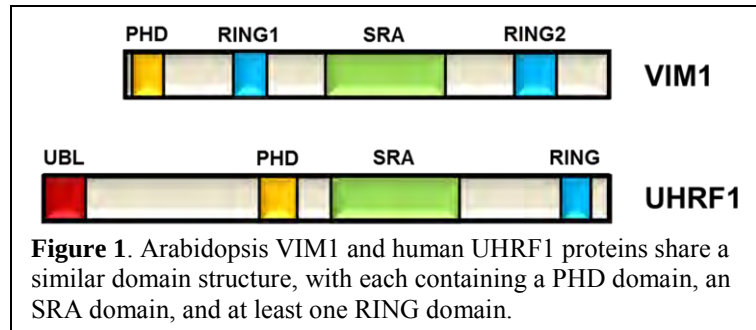
Task 1: Investigate the role of the VIM1 RING domain in epigenetic regulation in *Arabidopsis thaliana*

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)

This work is in early stages, with the main accomplishment so far being the creation of relevant plant lines. These were made in a *vim1* $-/-$ T-DNA insertion mutant background. I have transformed these 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 (Fig. 2). Thus, the only form of VIM1 expressed in these plant lines will be a version containing point mutations that should abolish RING domain activity.

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.



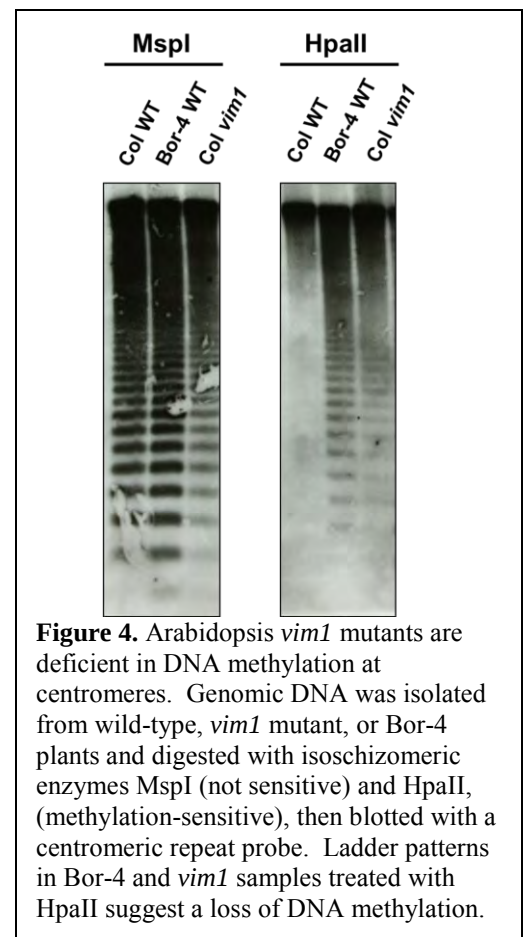
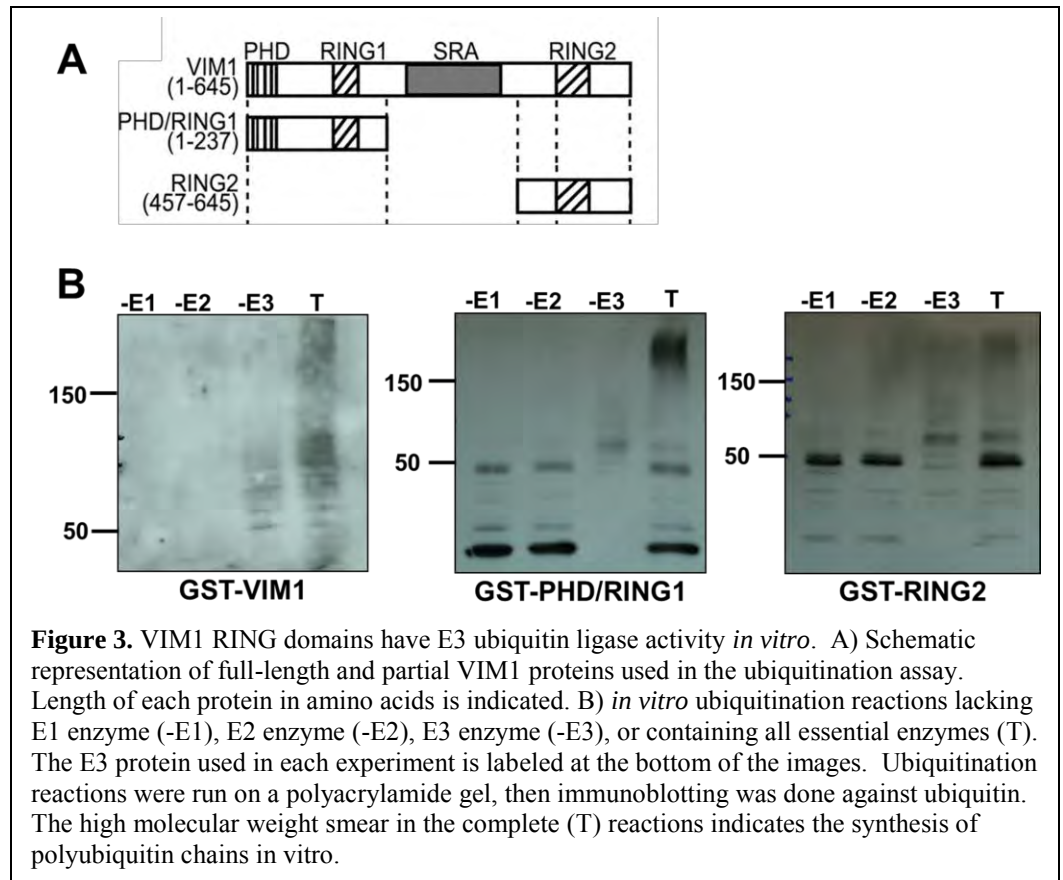
2. Confirm ubiquitination targets using an in vitro assay containing purified recombinant VIM1 (Months 1 – 36)

I have not yet completed the proteomics analysis described above, so there are no candidate substrates to test using an in vitro assay. However, I have developed an in vitro ubiquitination system that will be useful for these future experiments. In these assays, the required E1, E2, and E3 enzymes are combined in vitro under appropriate buffer conditions with other necessary components 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 vitro¹³. In addition, I have used partial recombinant fragments of VIM1 to show that each of the two individual RING domains has activity (Fig. 3).

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

My efforts to date in these experiments have been focused on generation of the RING domain point mutants described in Task 1, section 1 and Fig. 2. I will use the plant lines expressing these mutants to determine the relationship between VIM1 ubiquitin ligase activity and other epigenetic processes, such as maintenance of DNA methylation and heterochromatin structure. To examine DNA methylation, the Richards lab has previously used methylation-sensitive restriction digests combined with southern blotting against centromeric repeats (Fig. 4). Such an assay comparing DNA methylation between wild-type plants and those expressing VIM1 RING domain mutant proteins will provide insight on whether the RING domains are essential for VIM1's role in maintaining DNA methylation. 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 phenotype⁶.

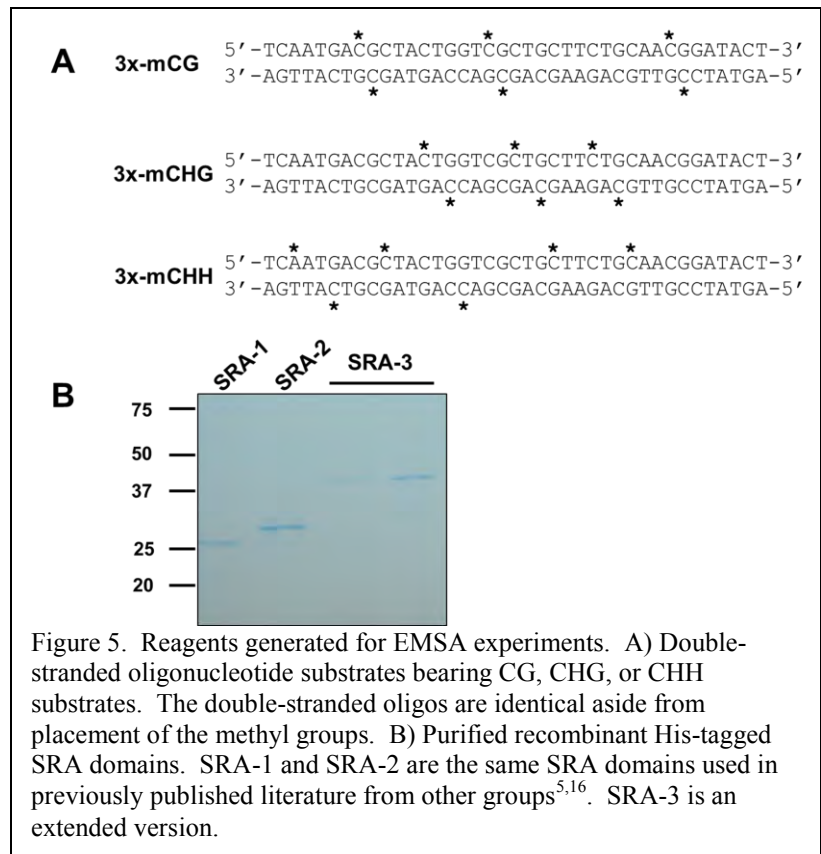
Task 2: Determine the *in vitro* specificities of the human UHRF1 RING and SRA domains

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 contexts⁵, 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. 5). We are currently optimizing the EMSA protocol using a commercial digoxigenin labeling and detection kit.



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.
- 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
- 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 (upcoming)

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.

REFERENCES

1. Novak, P. et al. Agglomerative epigenetic aberrations are a common event in human breast cancer. *Cancer Res* **68**, 8616-8625 (2008).
2. Hartmann, O. et al. DNA methylation markers predict outcome in node-positive, estrogen receptor-positive breast cancer with adjuvant anthracycline-based chemotherapy. *Clin. Cancer Res* **15**, 315-323 (2009).
3. Sinha, S. et al. Frequent alterations of hMLH1 and RBSP3/HYA22 at chromosomal 3p22.3 region in early and late-onset breast carcinoma: clinical and prognostic significance. *Cancer Sci* **99**, 1984-1991 (2008).
4. Rønneberg, J.A. et al. GSTP1 promoter haplotypes affect DNA methylation levels and promoter activity in breast carcinomas. *Cancer Res* **68**, 5562-5571 (2008).
5. Bostick, M. et al. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* **317**, 1760-1764 (2007).
6. Woo, H.R., Pontes, O., Pikaard, C.S. & Richards, E.J. VIM1, a methylcytosine-binding protein required for centromeric heterochromatinization. *Genes Dev* **21**, 267-277 (2007).
7. Wu, J. et al. Identification and functional analysis of 9p24 amplified genes in human breast cancer. *Oncogene* (2011).doi:10.1038/onc.2011.227
8. Tien, A.L. et al. UHRF1 depletion causes a G2/M arrest, activation of DNA damage response and apoptosis. *Biochem. J* **435**, 175-185 (2011).
9. Daskalos, A. et al. UHRF1-mediated tumor suppressor gene inactivation in nonsmall cell lung cancer. *Cancer* **117**, 1027-1037 (2011).
10. Alhosin, M. et al. Down-regulation of UHRF1, associated with re-expression of tumor suppressor genes, is a common feature of natural compounds exhibiting anti-cancer properties. *J. Exp. Clin. Cancer Res* **30**, 41 (2011).
11. Du, Z. et al. DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci Signal* **3**, ra80 (2010).
12. Karagianni, P., Amazit, L., Qin, J. & Wong, J. ICBP90, a Novel Methyl K9 H3 Binding Protein Linking Protein Ubiquitination with Heterochromatin Formation. *Mol. Cell. Biol.* **28**, 705-717 (2008).
13. Kraft, E., Bostick, M., Jacobsen, S.E. & Callis, J. ORTH/VIM proteins that regulate DNA methylation are functional ubiquitin E3 ligases. *Plant J* **56**, 704-715 (2008).
14. Barrès, R. et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab* **10**, 189-198 (2009).
15. Lister, R. et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462**, 315-322 (2009).
16. Qian, C. et al. Structure and hemimethylated CpG binding of the SRA domain from human UHRF1. *J Biol Chem* **283**, 34490-34494 (2008).