PI: **ibarra, christian Anthony**	Title: The Regulation of Gene Imprinting by Genome-Wide DNA Demethylation in Arabidopsis	
Received: 08/13/2009	FOA: PA09-209	Council: 01/2010
Competition ID: ADOBE-FORMS-A	FOA Title: Ruth L. Kirschstein National Research Service Awards for Individual Predoctoral Fellowships (F31) to Promote Diversity in Health-Related Research	
1 F31 GM093633-01	Dual: HG	Accession Number: 3220479
IPF: 577502	Organization: UNIVERSITY OF CALIFORNIA BERKELEY	
Former Number:	Department: Plant and Microbial Biology	
IRG/SRG: ZRG1 IMST-D (29)L	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A)	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Christian Ibarra	The Regents of the University of California, Berkeley	PD/PI
Robert Fischer	University of California, Berkeley	Faculty
Daniel Zilberman	University of California, Berkeley	Faculty

Reference Letters

[redacted]

Additions for Review

Other	Ibara eligibility letter
Updated Pages	Scholastic Section

APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	**State Application Identifier**

1. * TYPE OF SUBMISSION

☐ Pre-application ☐ Application ☒ Changed/Corrected Application

4. Federal Identifier GRANT10398435

5. APPLICANT INFORMATION

*** Organizational DUNS:** [redacted]

* Legal Name: The Regents of the University of California, Berkeley

Department: Sponsored Projects Office

Division:

* Street1: [redacted]

Street2:

* City: [redacted]

County: Alameda

* State: [redacted]

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: [redacted]

Person to be contacted on matters involving this application

Prefix:

* First Name: Deborah

Middle Name:

* Last Name: Rutkowski-Howard

Suffix:

* Phone Number: [redacted]

Fax Number: [redacted]

Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* [redacted]

7. * TYPE OF APPLICANT: H: Public/State Controlled Institution of Higher Education

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY:

National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

The Regulation of Gene Imprinting by Genome-Wide DNA Demethylation in Arabidopsis Endosperm

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*

CA-009

13. PROPOSED PROJECT:

* Start Date: 04/01/2010

* Ending Date: 04/01/2012

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant: CA-009

b. * Project: CA-009

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix:

* First Name: Christian

Middle Name:

* Last Name: Ibarra

Suffix:

Position/Title: Graduate Student Research

* Organization Name: The Regents of the University of California, Berkeley

Department: Plant and Microbial Biology

Division:

* Street1: [redacted]

Street2:

* City: [redacted]

County: Alameda

* State: [redacted]

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: [redacted]

* Phone Number: [redacted]

Fax Number:

* Email: [redacted]

OMB Number: 4040-0001
Expiration Date: 04/30/2008

6. Project Summary/abstract

DNA methylation (5-methylcytosine) in mammals and plants silences transposons, retroviruses, and regulates gene imprinting. In mammals, DNA hypermethylation is associated with certain diseases including the onset and progression of cancer. We discovered that the Arabidopsis DEMETER (DME) protein regulates imprinting by DNA demethylation. DME is related to DNA glycosylases that excise damaged/mispaired bases in the base excision DNA repair pathway. DME excises 5-methylcytosine that is replaced with cytosine. DME is expressed primarily in the central cell, the progenitor of the placenta-like endosperm that supports embryo development. DME demethylates and activates maternal allele expression of genes that are imprinted in the endosperm. In mammals, 88 imprinting genes have been discovered, many of which are crucial for proper embryo development. In contrast, only ten plant imprinted genes have been identified and studied in detail. To understand the genome-wide extent of DNA demethylation in the regulation of gene imprinting, we used the Illumina Genome Analyzer ™ to carryout high-throughput bisulfite sequencing on DNA from wild type endosperm, embryo and dme-mutant endosperm. In addition to finding several sites potentially regulated by DEMETER mediated DNA demethylation we also discovered how global DNA demethylation is invovled in trasposon silenicng.

7. PROJECT NARRATIVE

DNA methylation is an epigenetic modification that is critical for mammal development and aberrancies in it give rise to specific types of cancer. Interestingly, DEMETER is the only protein known to demethylate DNA, thusly reversing the epigenetic effects of DNA methylation. The potential applications for a protein with the ability to naturally demethylate DNA is important, particularly in the field of cancer diagnostics, and therefore demands the basic science required to elucidate the functions of it.

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Robert Fischer, University of California at Berkeley

Laboratory: Robert Fischer's laboratory facilities in Koshland Hall consist of approximately 1600 square feet of space with ample wet bench facilities. In addition, shared facilities include cold rooms, dark rooms, plant tissue culture rooms, and plant growth rooms.

Clinical: Not applicable.

Animal: Not applicable.

Computers: Three Mac and one Mac/PC computers, a laser printer, and a scanner are located in the laboratory. All are connected to the Ethernet and are linked by the UC Berkeley Internet service.

Office: Robert Fischer's office (170 square feet) is located adjacent to the laboratory and is equipped with a Mac computer for word processing.

Major Equipment: For the molecular biology aspects of the project, there are microcentrifuges, ultracentrifuges, preparative centrifuges, spectrophotometers, freezers, electrophoresis power supplies, personal computers, water baths, incubators, and bacterial growth chambers. For morphological analysis a Zeiss axiophot and axiovert microscopes for fluorescence, bright field, dark field, phase and DIC microscopy with computer image capture capability are available. In addition, the nearby Biological Imaging Facility is equipped with a cryotome, microtomes, fluorescence microscopes, a confocal microscope with computer-enhanced image analysis equipment.

Other Resources:

Greenhouse. Robert Fischer's lab uses 1000 square feet of a fully staffed greenhouse. The environment (temperature, light) of the greenhouse is computer controlled. There are also Percival plant growth chambers and walk in chambers for growing Arabidopsis plants under more precise conditions.

Daniel Zilberman, University of California at Berkeley

Laboratory: Daniel Zilberman's laboratory facilities in Koshland Hall consist of approximately 1000 square feet of space with ample wet bench facilities. In addition, shared facilities include cold rooms, dark rooms, plant tissue culture rooms, and plant growth rooms.

Clinical: Not applicable.

Animal: Not applicable.

Computers: The laboratory is equipped with six Intel-based quad-core workstations used for data analysis, five running a 64-bit implementation of Windows and one running Ubuntu Linux. Each Windows computer runs statistical analysis software (STATA), a graphical viewer for genomic data (SignalMap), a Perl interpreter, and Microsoft Office. The Linux workstation houses the web server. All computers are networked and connected to a color laser printer and a scanner. All genomic data are stored on a three terabyte RAID 5 fault-tolerant network disk array accessible from all computers in the Zilberman and Fischer laboratories.

Office: Daniel Zilberman's office (170 square feet) is located adjacent to the laboratory and is equipped with an Intel Core i7 quad-core 64-bit Windows workstation running statistical analysis software (STATA), a graphical viewer for genomic data (SignalMap), a Perl interpreter, and Microsoft Office.

Major Equipment: For the molecular biology aspects of the project, there are four gradient thermal cyclers, two microcentrifuges, a refrigerated preparative centrifuge, a vacuum concentrator, a Nanodrop spectrophotometer, a water bath sonicator, a Polytron tissue homogenizer, freezers, agarose and polyacrylamide electrophoresis tanks and power supplies, water baths, incubators, shakers, and a bacterial growth chamber.

Other Resources:

Vincent J. Coates Genomic Sequencing Laboratory. This core facility, which is housed on UC Berkeley campus in the newly constructed Stanley Hall, is equipped with two second-generation Illumina Genome Analyzers with paired end modules (http://www.qb3.org/gsl/Home.html). Each unit can produce over 4 billion bases of sequence in three days. Professional staff at the facility carry out cluster generation, sequencing and data extraction, and upload sequence files to an FTP server.

Greenhouse. Daniel Zilberman's laboratory uses 600 square feet of a fully staffed greenhouse. The environment (temperature, light) of the greenhouse is computer controlled.

A fully staffed greenhouse (1000 square feet) is available for use by Fischer lab research group. The environment (temperature, light) of the greenhouse is computer controlled. In addition, a plant growth room is available that is shared by one neighboring laboratory. Finally, we have three Percival chambers for growing plants .

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

For the molecular biology aspects of the project, there are ultracentrifuges plus rotors, preparative centrifuges plus rotors, spectrophotometers, scintillation counters, freezers, electrophoresis power supplies, personal computers, water baths, incubators, and bacterial growth chambers. For morphological analysis a Zeiss axiophot and axiovert microscopes for fluorescence, bright field, dark field, phase and DIC microscopy with computer image capture capability are available. The MALDI-TOF facility and staff at the UC Berkeley campus (http://biology.berkeley.edu/crl/mass_spec/index.htm) will assist us in protein mass spectrometery analysis. Array hybridization will be carried out by Nimblegen, Inc. (Madison, WI). Data analysis will be performed using the software platform provided by Nimblegen, Inc.

Section II – Sponsor (Robert Fischer) and Co-Sponsor (Daniel Zilberman) Information

1. Research Support Available

Current Research Support.

Funding Source and Number	Title	PI	Dates	Total Costs
National Institutes of Health, GM069415-05	Regulation and Function of DNA Demethylation in Arabidopsis	Fischer	6/1/08 – 4/30/12	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

Pending Research Support.

Founding Source	Title	PI	Dates	Total Costs
National Institutes of Health	Interconnection between DNA Methylation, H2A.Z, and Histone Binding Proteins	Zilberman	4/1/10 – 3/30/15	[redacted]
National Science Foundation	EMF-mediated Epigenetic Mechanisms in Arabidopsis	Zilberman and Sung	1/1/10 – 12/31/12	[redacted]

2. Fischer Previous Fellows/Trainees

A. Total number of predoctoral individuals previously sponsored. 14

B. Total number of postdoctoral individuals previously sponsored. 17

C. Five representative individuals:

Name	Present Employing Organization	Present Title
Robert Franks	Department of Genetics, North Carolina State University, Raleigh, NC 27695 USA	Assistant Professor
Yuki Mizukami	Biology Department, Purdue University, West Lafayette, IN 47907 USA	Assistant Professor
Yeonhee Choi	Biology Department, Seoul National University, Seoul, Korea	Associate Professor
Ramin Yadegari	Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721 USA	Associate Professor
Nir Ohad	Department of Plant Sciences, Tel-Aviv University, Tel-Aviv, 69978, Israel	Associate Professor

2. Zilberman Previous Fellows/Trainees

A. Total number of predoctoral individuals sponsored. 2

B. Total number of postdoctoral individuals sponsored. 2

C. Representative individuals: One postdoctoral fellow has left Daniel Zilberman's lab.

Name	**Present Employing Organization**	**Present Title**
Leor Eshed-Williams	Hebrew University of Jerusalem, Faculty of Agriculture, Jerusalem, Israel	Assistant Professor

3. Training Plan, Environment, Research Facilities

The laboratories of Robert Fischer (sponsor) and Daniel Zilberman (co-sponsor) will provide Christian Ibarra with an excellent research environment. The primary research goal of Robert Fischer's lab is to understand how DNA demethylation controls gene imprinting and endosperm development. Since 2002, his lab has published ten manuscripts (2 in Cell, 2 in Science, Developmental Cell, 2 in PNAS, Plant Cell, 2 in Plant Physiology) and five invited reviews on imprinting and chromatin dynamics. Christian Ibarra also interacts with Daniel Zilberman, whose research goal is to understand the regulation of transcription within the context of chromatin, which is influenced by transcription factors, nucleosomes, chromatin remodelers, histone variants and modifications, and DNA methylation. Daniel Zilberman has an excellent publication record, and has published 6 outstanding papers (Nature, Science, 2 in Nature Genetics, PNAS, Development) in the past 3 years. Robert Fischer and Daniel Zilberman will provide the necessary equipment, biological materials, supplies and training expertise to help Christian Ibarra achieve his research and career goals. Robert Fischer will provide mentoring on gene imprinting and endosperm development, whereas Daniel Zilberman will provide mentoring in chromatin biology and the analysis of genome-wide data sets. This collaboration is already well underway, and has resulted in a co-first author publication for Christian Ibarra (Genome-wide demethylation of Arabidopsis endosperm, ***Science*****,** 324: 1451-1454 (2009)), as well as an NSF grant to study gene imprinting networks in Arabidopsis.

UC Berkeley provides a highly stimulating academic environment for predoctoral fellows. Christian Ibarra will take classes to enhance his understanding of genetics and genome analysis (i.e., MCB 240, Advanced Genetic Analysis; MCB/PB C246, Topics in Computational Biology and Genomics). Also, the Molecular and Cell Biology Department, Plant Biology Department, and the Graduate Group in Computational and Genomic Biology sponsor seminars in the fields of genetics, genomics and computational biology.

The research program proposed by Christian Ibarra will complement his knowledge of genetics and molecular biology and extend his understanding of epigenetic mechanisms that control gene imprinting and chromosome architecture. He will have an opportunity to become facile in the use of genome-wide DNA sequencing platforms, microarray technology, software, and statistical methods used to study DNA methylation. These approaches will allow him test his hypotheses about how DNA demethylation controls gene imprinting. He will be working with scientists in my lab and in Daniel Zilberman's lab who share his enthusiasm for epigenetics and can provide new approaches and paradigms for his studies.

Robert Fischer's laboratory consists of 1600 square feet of modern laboratory space in Koshland Hall at UC Berkeley. For molecular biology experiments, there are PCR machines, spectrophotometers, scintillation counters, freezers, electrophoresis power supplies, computers, incubators, and growth chambers. Zeiss axiophot and axiovert microscopes for fluorescence, bright field, dark field, phase and DIC microscopy with computer image capture capability are available for analyses of transgenic and mutant plants. To grow plants we use a fully staffed greenhouse as well as growth chambers. Daniel Zilberman's laboratory is on the same floor of Koshland Hall, consists of 1000 square feet with ample wet bench space, and is equipped with nine Intel-based quad-core workstations used for data analysis, five running a 64-bit implementation of Windows and four (a computer cluster) running Ubuntu Linux. Each Windows computer runs statistical analysis software (STATA), a graphical viewer for genomic data (SignalMap), a Perl interpreter, and Microsoft Office. The Linux workstations house the web server and computational pipeline. All computers are networked and connected to a color

laser printer and a scanner. All genomic data are stored on a three terabyte RAID 5 fault-tolerant network disk array accessible from all computers in the Zilberman and Fischer laboratory.

4. Number of Fellows/Trainees to be Supervised During the Fellowship.

In addition to Christian Ibarra, the Sponsor (Fischer) and Co-Sponsor each supervise one predoctoral and two postdoctoral trainees.

5. Applicant's Qualifications and Potential for a Research Career

Christian Ibarra has been a graduate student in Robert Fischer's laboratory for over two years. His main interest has been to understand how gene imprinting is regulated by DME-mediated DNA demethylation. Christian uniquely tackled the problem on a genome-wide basis. He was a catalyst for the collaboration between my lab and Daniel Zilberman's lab. Christian is thriving in this collaborative research environment. He spends long hours thinking about his research, and extensively consults with Daniel Zilberman and me, as well as graduate students and postdoctoral fellows in both labs. Christian has an excellent understanding of the principles of molecular biology and genetics. He is very intelligent and enjoys reading the current scientific literature. He asks many penetrating questions and is highly motivated to advance his research project. He works long hours in the lab and diligently studies for his classes. Christian develops, expresses, and tests his own hypotheses about his research. He is well organized and carefully plans his research and study time. Christian Ibarra is also highly motivated to teach. He gives well-organized and interesting seminars at our laboratory meetings. On his own volition, he has arranged to give presentations to high school students, particular to students who are underrepresented minorities in the sciences. In summary, I give Christian Ibarra my highest recommendation for an NIH predoctoral fellowship He is comparable to the best graduate students who have worked in my lab these past 20 years. I am sure he will develop an exciting research program, be an inspirational teacher, will graduate with distinction, and will go on to generate his own successful career in research.

A. AIMS. The goal of this work is to elucidate the role of DEMETER-mediated DNA demethylation in early endosperm development and plant gene imprinting.

SPECIFIC GOALS. I will address the following questions about the regulation of gene imprinting.

1. How does genome-wide DNA demethylation regulate genomic imprinting?

2. Did genomic imprinting arise as a byproduct of silencing transposons that insert into the genome?

3. Did global demethylation in the central cell, resulting in gene imprinting, evolve to ensure the silencing of transposable elements in the embryo?

B. BACKGROUND AND SIGNIFICANCE

1. The importance of DNA methylation in plants and mammals. DNA methylation is the modification of DNA by the addition of a methyl group to the 5-carbon of cytosine, which is carried out by a family of enzymes called methyltransferases. In both mammals and plants, DNA methylation serves as an epigenetic mark that recruits chromatin remodeling and modifying enzymes, allowing for the formation of heterochromatin and subsequent transcriptional silencing. In mammals, DNA methylation controls important process such as genomic imprinting, X-chromosome inactivation, and the silencing of repetitive DNA elements. In plants, DNA methylation it is responsible for imprinting and transposon repression (1,2).

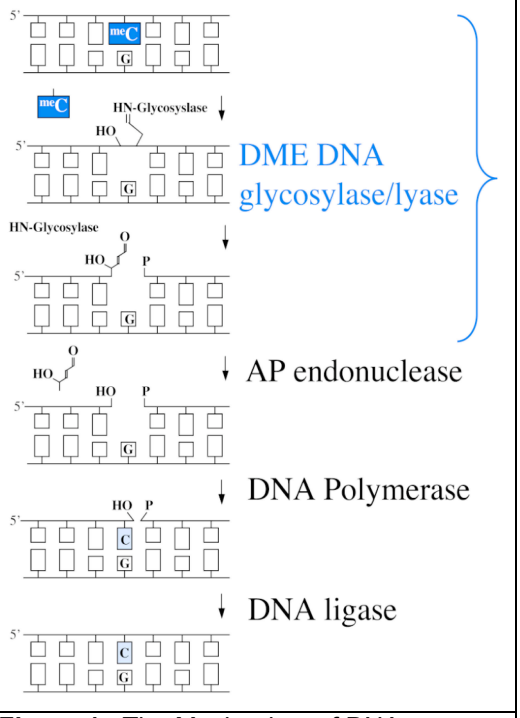

Figure 1. The Mechanism of DNA demethylation in *Arabidopsis*

2. DNA demethylation and the base excision repair pathway. Enzymatic DNA demethylation has been shown to occur in plants (18). In *Arabidopsis*, active DNA demethylation is carried out by the DEMETER (DME) family of bifunctional helix-hairpin-helix DNA glycosylases that have both DNA glycosylase and apurinic/apyrimidinic (AP) lyase activity (4,5). DNA glycosylases are part of the base excision repair pathway (BER), enzymes that recognize specific lesion bases in DNA and remove them (Figure 1). The resulting abasic lesions are then further processed by endonucleases, a repair polymerase and a ligase, ultimately to effect complete restoration of the original DNA sequence (6). The DEMETER family of enzymes have co-opted the function of the BER pathway to excise 5-methylcytosine, instead of mismatched or aberrant base pairing, enabling them to demethylate DNA (Figure 1). The DEMETER family of DNA glycosylases in *Arabidopsis* is comprised of DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2), and DEMETER-LIKE 3 (DML3), all capable of active DNA demethylation. In animals, recent studies have shown compelling evidence for the presence of a similar active DNA demethylation mechanism (37,38).

3. DEMETER-mediated DNA demethylation and genomic imprinting. Imprinting is the differential expression between two alleles of a gene in a parent-of-origin specific manner, and has been observed in both mammals and plants, playing an important role in their reproductive strategies (22). Occurring in the placenta of mammals and the endosperm of flowering plants, imprinted genes tend to be expressed in these structures, which serve as a conduit for the essential flow of nutrients from the maternal parent to the embryo.

In *Arabidopsis*, DME establishes the imprinting of two Polycomb group genes (*MEDEA* (*MEA*) and *FERTILZATION-INDEPENDENT SEED2* (*FIS2*)) and a transcription factor gene (*FLOWERING WAGENINGEN* (*FWA*)) by specifically demethylating and activating the expression of the maternal allele, resulting in imprinted expression (14). This occurs specifically in the central cell of the female gametophyte. In plants, two sperm cells fertilize the central and egg cells resulting in the formation of endosperm and embryo, respectively. The endosperm is a terminally differentiated tissue that provides nutrients to the embryo. DME is expressed primarily in the central cell where it demethylates the maternal genome (5, 12). Moreover, DME expression is critical for proper seed development,

since *Arabidopsis* lines with a mutant maternal *dme* allele have a striking phenotype in the form of aborted, inviable seed.

4. Regulation of gene imprinting by DME. The DME DNA glycosylase activates *MEA*, *FIS2*, and *FWA* maternal allele expression in the *Arabidopsis* central cell (Figure 2) (5,29,39). Expression of *FWA* is silenced by DNA methylation maintained by the MET1 DNA methyltransferase. In the central cell DME removes DNA methylation at the promoter of the maternal *FWA* allele. DME is not expressed in sperm cells so the paternal FWA allele remains methylated and silenced. Upon fertilization of the central cell with a sperm nucleus, DME expression is dramatically reduced (12). As a result, only the maternal *FWA* allele is expressed in the endosperm. Thus, *FWA* imprinting is established by maternal central cell specific gene activation by the DME DNA glycosylase.

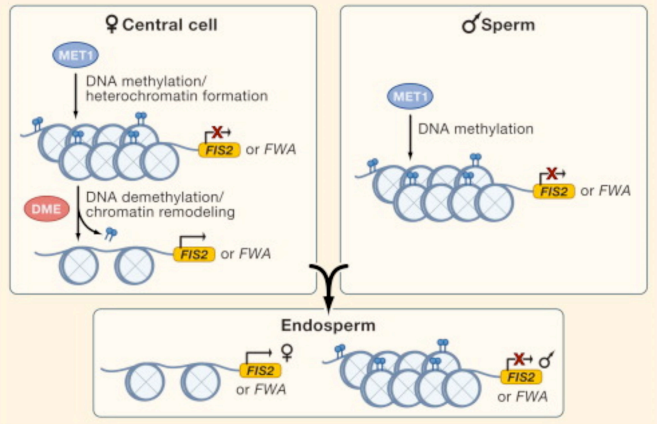

Figure 2. The mechanism for imprinting of the *FWA* and *FIS2* genes in *Arabidopsis.*

5. The RNA-directed DNA methylation pathway in plants. RNA-directed DNA methylation (RdDM) is a process in which small interfering RNAs (siRNAs) direct the methylation of DNA sequences that are complementary to siRNAs (40). These siRNAs are 21-24 nt in length and originate from dsRNA generated by bidirectional transcription, extended fold backs with perfect complementarity, or RNA-dependent RNA polymerase (RDRP)-based mechanisms (41). Upon generation of the precursor dsRNA, a Dicer-like enzyme processes the dsRNA generating siRNAs, which are then loaded on to an Argonaute 4-containing RNA-induced silencing complex (RISC). Primed with siRNAs, the RISC complex then associates with DNA methyltransferases specific for *de novo* methylation (CHROMOMETHYLASE 3 and DOMAINS REARRANGED METHYLTRANSFERASE) (43,44) and methylates the target DNA sequences by way of sequence complementarity. In this way, siRNAs serve as both a primary signal for maintaining most of the non-CG methylation in the plant genome, and are a critical component of the RdDM pathway. In *Arabidopsis*, over 100,000 different siRNAs exist, which are believed to be transcribed from thousands of loci (35)

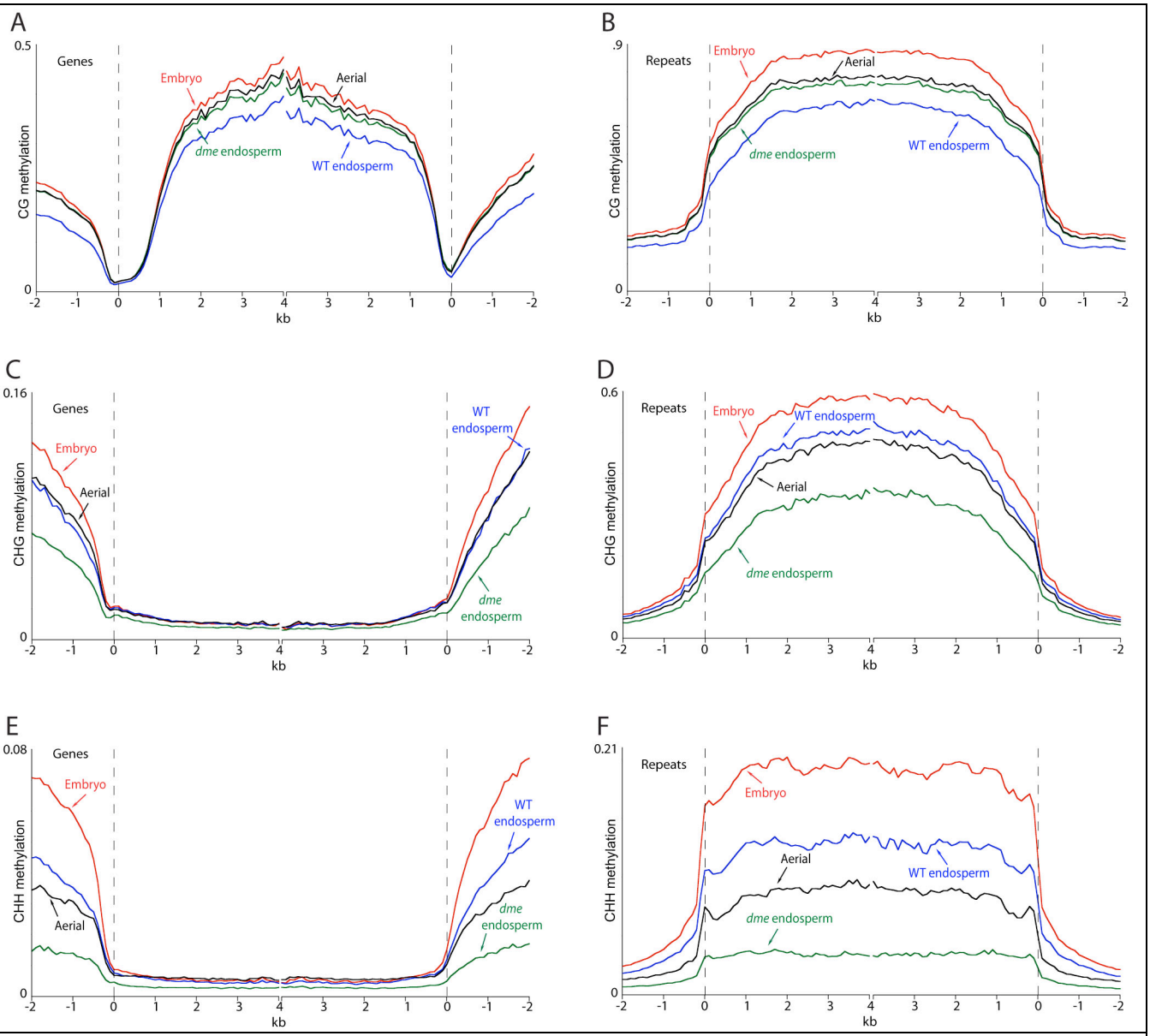

Figure 3. Profiles of DNA methylation in embryo, wild-type endosperm, and dme endosperm. **(A to F)** TAIR8-annotated genes [**(A)**, **(C)**, and **(E)**] or transposons [**(B)**, **(D)**, and **(F)**] were aligned at the 5' end (left panel) or the 3' end (right panel), and average methylation levels for each 100-bp interval are plotted from 2 kb away from the gene (negative numbers) to 4 kb into the gene (positive numbers). Embryo methylation is represented by the red trace, wild-type (WT) endosperm by the blue trace, *dme* endosperm by the green trace, and aerial tissues by the black trace. The dashed line at zero represents the point of alignment. CG methylation is shown in **(A)** and **(B)**, CHG in **(C)** and **(D)**, CHH in **(E)** and **(F)**.

6. Genome-wide DNA demethylation in the *Arabidopsis* endosperm. To understand the role of DME-mediated DNA demethylation in the *Arabidopsis* seed, we recently carried out a comprehensive genome-wide analysis of DNA methylation in the *Arabidopsis* endosperm, embryo and *dme*-mutant endosperm (27). We determined the methylation landscape of these genomes using the technique of bisulfite conversion coupled to genome-wide sequencing with the Illumina Genome Analyzer™ platform. This approach, compared to using immunoprecipitation with 5-methylcytosine antibodies, has the significant advantage of distinguishing the DNA methylation contexts found in plants (CG, CHG and CHH), ultimately allowing for the highest resolution analysis of the endosperm and embryo methylomes. As shown in Figure 3, we discovered that virtually the entire endosperm genome is demethylated, coupled with extensive local non-CG hypermethylation of siRNA-targeted sequences.

Moreover, endosperm demethylation is accompanied by CHH hypermethylation of embryo transposable elements. We also found that the *dme*-mutation has the effect of partially restoring endosperm CG methylation to levels found in other tissues, suggesting that CG demethylation is specific to maternal sequences. Our findings demonstrate extensive reconfiguration of the endosperm methylation landscape.

7. Significance. Plants and animals have common epigenetic mechanisms that play vital roles in health. In both plants and animals, DNA methylation is established and maintained by related DNA methyltransferases (48), DNA is demethylated by DNA repair enzymes (14, 37, 38), and aberrant DNA methylation causes disease. The research program outlined in this proposal will elucidate the molecular mechanisms for DNA demethylation, a process that regulates both gene imprinting and disease resistance in plants and mammals. DNA hypermethylation at specific loci is associated with the onset and progression of cancer and may be an important target for diagnosis and treatment (49, 50). Demethylation by DNA glycosylases may be an approach that may be used in the long term to reverse harmful DNA hypermethylation. Understanding the mechanism of DME-mediated DNA demethylation may enable us to development more efficient diagnostic tests for the DNA hypermethylation associated with the onset and progression of cancer. Alterations in patterns of DNA methylation are also associated with congenital diseases due to defects in gene imprinting (51-62). Understanding the mechanisms of genetic imprinting and DNA demethylation will provide important insights into these diseases, as well.

C. PRELIMINARY STUDIES. All my preliminary results were recently published (Hsieh, T.-F., Ibarra, C., et al (2009) Genome-Wide Demethylation of *Arabidopsis* Endosperm. *Science* 324:1451-1454.

D. RESEARCH DESIGN AND METHODS

1. How does genome-wide DNA demethylation regulate genomic imprinting?

i) Rationale. DME-mediated DNA demethylation regulates and imprints the gene expression of eight genes *MEDEA*, *FIS2*, *FWA, HDG9, HDG8, HDG3, ATMYB3R2,* and *AT5G62110* in the central cell (5,14,28,29). However, our results suggest that imprinted genes are not exceptional sequences specifically targeted for demethylation in the central cell. Rather, they appear to be part of a nearly universal process that reshapes DNA methylation of the entire maternal genome in the endosperm. Imprinted expression of genes regulated by allele-specific DNA methylation could potentially arise whenever a transposable element insertion or a local duplication near a gene's regulatory sequences induces methylation and gene silencing in other tissues, including the paternal endosperm genome.

Since DME is only active in the central cell, comparing genome DNA methylation profiles between wild type endosperm and embryo should reveal differentially methylated regions (DMRs), loci that are potential targets of DME-mediated DNA demethylation. In particular, DME target loci would be hypomethylated in the wild type endosperm compared to the embryo. Likewise, this hypomethylation would be lost in the *dme*-mutant endosperm. After selecting DMRs based upon these criteria, we discovered that genes exhibiting reduced endosperm (compared to embryo) methylation upstream of the start of transcription were, as a group, preferentially expressed in the endosperm compared to the embryo (27). These analyses suggested 1097 genes could potentially be imprinted in the *Arabidopsis* endosperm. As described in the experiments below, I will select candidate genes for analysis, identify new imprinted genes, and help elucidate how genome-wide DNA demethylation regulates gene imprinting.

ii) Identifying candidates for parent-of-origin expression. I will first select genes with DMRs associated with well-defined transposable elements. Within this group, I will further select those genes that are silenced not only in the embryo, but are silenced throughout the adult tissues of the plant. I expect that this will reduce the number of potentially imprinted genes to a manageable number (e.g., 200).

iii) Determine if candidate imprinted genes exhibit parent-of-origin specific expression and methylation. To determine if these candidate imprinted genes from our deep-sequencing analysis are truly imprinted, I will carry out allele-specific bisulfite sequencing to validate the sites of DNA demethylation. This will be accomplished using procedures that the Fischer lab has successfully used in the past to analyze allele-specific DNA demethylation of *MEA* by DME in the endosperm (5). That is, ecotypes with SNP polymorphisms will be crossed, F1 endosperm will be isolated, DNA purified, bisulfite treated, and sequenced by the Sanger method. I will also determine if the genes are imprinted, and if *dme* mutations affect their imprinted status in the endosperm. For these experiments, I will use allele-specific RT-PCR amplification procedures used by the Fischer lab to measure allele-specific *MEA* expression in wild type and mutant genetic backgrounds (5).

iv) Examine the biological function of candidate imprinted genes. The predicted molecular function of each candidate imprinted gene will be assessed using the Arabidopsis Gene Ontology (GO) resource (http://www.arabidopsis.org/portals/genAnnotation/functional_annotation/go.jsp). To further assess their biological functions, I will also search the collections of T-DNA mutagenized *Arabidopsis* lines for mutant alleles of the imprinted genes. The collections are available at the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). The effect of lesions of the

newly identified imprinted genes on endosperm growth, development, and seed viability will be assessed. This will be accomplished by morphological visualization of cleared seeds, quantifying the number of nuclear cytoplasmic domains in the endosperm, measuring the timing of cellularization, and analyzing the expression of reporter genes (*GFP* and *GUS*) transcribed by endosperm marker promoters (12,15-17).

2. Did genomic imprinting arise as a byproduct of silencing transposons that insert into the genome?

i) Rationale. In *Arabidopsis*, DNA methylation is critical for the silencing of transposable elements, which if expressed and are allowed to transpose, could deleteriously disrupt the genome. Our results suggest that imprinted genes are correlated with being adjacent to upstream silenced transposons. To test this hypothesis, I will create transgenic plants bearing transgenes with reporters in different configurations relative to transposons. I will then determine which configurations lead to imprinting of the reporter gene.

ii) Construction of transgenes. I will choose to use the two transposons that are the most commonly found next to the imprinted genes I have analyzed in the section above. The reporter gene will encode the green fluorescent protein (GFP). The promoter for the *GFP* gene will be the constitutively expressed, non-imprinted ubiquitin (*UBQ*) promoter (45). I will create transgenes with the transposon at 500 base pair intervals up to 3 kb upstream of the *UBQ:GFP* transgene. I will likewise create a series of control constructs with the transposon downstream of the *UBQ:GFP* transgene. The constructs will also have a selectable marker (resistance to the Basta herbicide) in order to obtain transformants. Constructs will be transformed into wild type *Arabidopsis* plants using the floral dip method. Transgenic plants will be selected on MS-plates containing Basta, and transferred to soil for continued growth.

(iii) Analysis of transgenic plants and expectations. If my hypothesis is correct, I expect to see transposon silencing of GFP in all the tissues except in the central cell and endosperm. Mature unfertilized ovules will be obtained and assessed for the presence of *GFP* expression in the central cell using fluorescence microscopy. Lines that show *GFP* expression in the central cell will be reciprocally crossed to wild-type (non-transgenic) plants, and allele-specific *GFP* expression will be examined in F1 endosperm. To determine that the silencing of the GFP reporter is due to DNA methylation at the transposon, I will also bisulfite sequence the construct in selected transformed lines.

(iv) Controls and alternative strategies. As a control, I will also use a construct with only *UBQ:GFP* and no transposon. I expect this to be expressed constitutively in endosperm, embryo, and throughout the plant. It is also possible that the *UBQ* promoter will be too strong to be silenced by DNA methylation from an adjacent transposon. If this appears to be the case, I will use the promoter for the α*-VPE* (vacuole processing enzyme) gene that is known to be weakly biallelically expressed in the endosperm and embryo (5).

3. Did global demethylation in the central cell, resulting in gene imprinting, evolve to ensure the silencing of transposable elements in the embryo?

(i) Rationale. Our results suggest a model where initially the central cell genome is demethylated. This global demethylation removes the methylation-mediated transposon suppression system, which results in the activation of transposable elements and the accumulation of siRNAs. A

strikingly different genome landscape is found in the embryo, where DNA methylation levels remain significantly high compared to endosperm, and various other types of adult tissues (27, Figure 3). Thus, although the endosperm and embryo are siblings within the seed, they appear to have opposite epigenetic states in regards to their genomes. Such epigenetic differences between the two genomes maybe a reflection of their fates, as the endosperm genome is not transmitted to the next generation, while the embryo genome is. The endosperm genome can tolerate transposon movement activated by global DNA demethylation, since it will not have its heritable material passed on to the next generation. However, the embryo cannot tolerate extensive transposon movement, as the contents of its genome (gametes) will be inherited by the next generation. The maintenance of posterity and structural integrity of the embryo genome is of utmost importance for the plant and one could speculate that almost certainly a mechanism would have evolved that enhances and supports the integrity of the embryo genome. Indeed, it may be that global demethylation in the central cell occurs in order to ensure the silencing of transposable elements in the embryo**.** It could be that siRNAs produced by demethylating the central cell genome are transported into the embryo and then carryout silencing of transposable elements in the embryo by the RdDM pathway. Recent work by Slotkin et al suggests such a scenario exists between the genomes of the non-reproductive vegetative cell and heritable sperm cells of the pollen (36). I will carry out the following experiments to test the hypothesis that siRNAs generated in the central cell move and silence in the embryo.

(ii) Construction of transgenes and transgenic plants. In order to test whether a siRNA can move from the central cell to the embryo I will first design an artificial miRNA targeting the *GFP* transcript (*amiRNA-GFP*) as described by Slotkin et al (36). The *amiRNA-GFP* sequence will be expressed under the control of a central cell specific promoter, *AGL61* (46, 47). The *AGL61:amiRNA-GFP* construct will be transformed by the floral dip method into plants homozygous for *DD45:GFP*, which expresses *GFP* specifically in the egg cell (42). The *DD45:GFP* line and the *AGL61* promoter are available from our collaborator on other projects (Dr. Gary Drews, U. Utah)**.** Transgenic lines (T1) will be obtained and unfertilized ovules will be examined for the presence of GFP activity in the egg cells using fluorescent light microscopy.

(iii) Expectations. If the miRNA (*amiRNA-GFP*) can move from the central cell to the egg cell, then plants that are homozygous for *DD45:GFP* and heterozygous for *AGL61:amiRNA-GFP* would be expected to have 50% ovules with fluorescent egg cells and 50% ovules without a fluorescent egg cell, due to silencing by *amiRNA-GFP*. As a negative control experiment, I will transform the *amiRNA-GFP* without a promoter, which should not silence *DD45:GFP*. As a positive control, I will create a *DD45:amiRNA-GFP* construct and transformed it into homozygous *DD45:GFP* plants, which should silence the *DD45:GFP* expression without the requirement of *amiRNA-GFP* movement.

4. Courses and training to assist my completion of the project. To facilitate my research and to assist my genome-wide analysis of imprinted genes in Arabidopsis, I will attend basic scripting languages classes such as Perl and Python, which are essential for the analysis of genomic data sets. I will take classes to enhance my understanding of genetics and genome analysis (i.e., MCB 240, Advanced Genetic Analysis; MCB/PB C246, Topics in Computational Biology and Genomics). Also, the Molecular and Cell Biology Department, Plant Biology Department, and the Graduate Group in Computational and Genomic Biology sponsor seminars in the fields of genetics, genomics and computational biology. In addition, I will strengthen my statistical analysis background by taking advanced statistics classes. Trainings on microscopy and advanced molecular biology skills are available in the Department of Plant and Microbial Biology at UC Berkeley. Learning of genomic techniques and analyses skills will primarily come from my personal interactions with Dr. Zilberman

and his bioinformatics specialist Pedro Silva, as well as Dr. Fischer and members in both laboratories.

5. Time table and work plan.

	Goal 1	Goal 2	Goal 3
Year 1	1. Identify candidate imprinted genes 2. Validate methylation status and examine their parent of origin expression status.	1. Identify top two most commonly TEs adjacent to candidate imprinted genes.	1. Construct *AGL61:amiRNA-GFP* transgene and transgenic lines. 2. Obtain *DD45:GFP* lines.
Year 2	1. Order T-DNA knockout lines from The Arabidopsis Stock Center. 2. Examine phenotypes of T-DNA mutants of new imprinting genes	1. Engineer *TE-UBQ:GFP* transgene series and transgenic plants. 2. Analyze transgenic lines GFP expression in the central cell.	1. Transform *AGL61:amiRNA-GFP* into *DD45:GFP* lines to obtain double transgene lines 2. Examine the effect of miRNA generated from the central cell on the *DD45:GFP* expression in the egg cell.
Year 3	1. Continue to examine T-DNA mutant lines.	1. Study parent of origin expressing of *TE-UBQ:GFP* lines by reciprocal crossing to wt plants.	

I. RESPECTIVE CONTRIBUTIONS

I, with help from my thesis advisor, Robert Fischer, and Daniel Zilberman who serves on my Thesis Committee, designed this research project. During laboratory meetings and conversations, we discussed goals and methods of this proposal.

J. SELECTION OF SPONSORS AND INSTITUTION

During my first year of graduate school in the Department of Plant and Microbial Biology at the University of California Berkeley, I attended a seminar that was given by Dr. Robert Fischer and was enthralled by his work on DNA demethylation and genomic imprinting. I subsequently did a rotation in his lab, upon which I then joined the lab, thus making him both my thesis advisor and mentor. In particular, I am devoting my graduate school studies to investigating the functions of the DME protein and its ability to regulate gene expression by DNA demethylation. I believe my work will advance knowledge in the fields of epigenetics and genomic imprinting. Moreover, I find the tutelage of Dr. Fischer exceptional and the expertise of the lab unmatched, as evident by a consistent publication record in top peer-reviewed journals. About a year later, Daniel Zilberman joined the faculty at UC Berkeley. In our discussions, I came to believe that significant insights into the regulation of gene imprinting could be obtained if we studied the process of DNA demethylation on a genome-wide basis. Daniel has graciously served as a second thesis advisor for me and helped me to attain my research goals. I attend his weekly lab meetings and interact frequently with his graduate students and postdoctoral fellows. In this way I am being trained in the fields chromatin biology and genomics.

The University of California Berkeley is one of the premier places to train as a graduate student. The faculty and staff in the Department of Plant and Microbial Biology not only care about the education and training of their graduate students, but also strive to help them further their careers. In addition to this, there are several outstanding core facilities which are expertly staffed and equipped, providing graduate student with excellent resources. For my research proposal, I will be frequently using the DNA sequencing facilities (Vincent J. Coates Genomic Sequencing Laboratory in Stanley Hall for high-throughput sequencing and the Sanger Sequencing Facility in Barker Hall). The proposed work will provide me with training in several state-of-the-art biological and molecular techniques, which is critical for my scientific career.

K. RESPONSIBLE CONDUCT OF RESEARCH

As a graduate student I am required to attend a Responsible Conduct of Research seminar and course for credit. I will be taking this seminar and course in the up-coming fall 2009 semester. This course consists of case studies presented by faculty and is conducted on a weekly basis. The faculty leads a discussion on the different points of ethical concern, within each case, which we will discuss as a group. The legal points are described for each scenario and we will be instructed as to whom to contact if such events were to occur.

8. GOALS FOR FELLOWSHIP TRAINING AND CAREER

Ultimately, my goal is to be a scientist in an academic research laboratory and the research in this proposal will provide me with superb training in the fields of genetics, molecular biology, and genomics. Experimental design, trouble-shooting, and execution will also be learned. Molecular biology tools that will be learned include RNA and DNA isolation, basic and advanced PCR techniques, cloning, and bacterial and plant transformation. Genomic tools that will be learned include building DNA libraries specific for deep-sequencing and various programs for data analysis. In addition, other projects in my lab will provide exposure to the fields and techniques of biochemical and chromatin research.

This proposal also contains many career development experiences. A number of conferences will be attended including ones where seminars, posters, or both will be presented. These conferences cover both broad and specific areas of study and provide avenues for networking. There will be a number of opportunities to write and publish work as well.

9. ACTIVITIES PLANNED UNDER THIS AWARD

21. ACTIVITIES PLANNED UNDER THIS AWARD: Approximate percentage of proposed award time in activities identified below. *(See instructions.)*

	Year	Research	Course Work	Teaching	Clinical
	First	70	30	0	N/A
	Second	50	0	50	N/A
	Third	100	0	0	N/A
	PREDOCTORAL FELLOWSHIPS ONLY				
	Fourth	100	0	0	N/A
	Fifth	100	0	0	N/A
	MD/PhD FELLOWSHIPS ONLY				
	Sixth				

Briefly explain activities other than research and relate them to the proposed research training.

The Doctoral Dissertation And Other Research Experience.

Undergraduate research experience (1999 – 2002). I first became interested in biological research as a media technician in a small biotech start-up company called Teknova Inc. Already a declared Cell and Molecular Biology major, I was fascinated by science and sought to gain practical experience by working in a laboratory. Responsible for making many different types of buffers, reagents and Petri dishes, it was at Teknova Inc. that I first became familiar with the modern biological research laboratory. To gain further experience in academic research, I searched for opportunities at San Francisco State University and obtained a position as an undergraduate researcher in the laboratory of Dr. Leticia Márquez-Magãna. Her microbial genetics laboratory examines bacterial motility, and the aim of my project was to determine the relationship between nutritional regulation and the activity of the flagellar gene in the Gram-positive bacteria Bacillus subtilis. Using a β-galactosidase reporter for this gene, we were able to show that an important global motility regulator, CodY, repressed flagellar gene expression in the presence of branch chain amino acids. This work led to the culmination of my first peer reviewed scientific paper: *Bergara, F., Ibarra, C, Iwamasa, J., Patarroyo, J.C., Aguilera, R., Marquez-Magana, L.M. CodY is a nutritional repressor of flagellar gene expression in Bacillus subtilis. J Bacteriol. 2003 May;185(10):3118-26.*

Professional research experience (2002 – 2004). Upon graduation, I took a job at the University of California San Francisco as a Staff Research Associate in the laboratory of Drs. Lily and Yuh-Nung Jan. This is an exceptionally large neuroscience laboratory consisting of over forty researchers. I worked alongside post-doctoral scientists investigating the function of the G protein-coupled inwardly rectifying potassium channel 3.2 (GIRK2). Our approach was to generate a library of randomly mutagenized GIRK2 in yeast via a technique called DNA shuffling and then identify candidate channels defective for GIRK2 function. RNA encoding the affected candidate channels was then made by in vitro transcription, and injected into Xenopus oocytes resulting in expression of the mutant channel on the oocyte surface. A functional assay was then conducted using two-electro voltage clamp and patch-clamp recording techniques, measuring the channel's permeability as well as selectability for K+. Our work resulted in the determination of several critical amino acids involved in GIRK2 potassium permeability and selectivity, and led to another peer reviewed publication: *Bichet D, Lin YF, Ibarra CA, Huang SH, Yi AB, Jan YN, and Jan LY. Evolving potassium channels by means of yeast selection reveals structural elements important for selectivity. Proc Natl Acad Sci U S A. 2004 Mar 30;101(13):4441-6.*

Doctoral Dissertation (in progress) at UC Berkeley (2006 – current). For my doctoral dissertation, I am focusing on how parent-of-origin-specific (imprinted) gene expression is regulated in Arabidopsis thaliana endosperm. I have discovered that cytosine demethylation of the maternal genome, mediated by the DNA glycosylase DEMETER, plays a critical role in regulating gene imprinting. Mentored by Professors Robert Fischer and Daniel Zilberman, I found that virtually the entire endosperm

genome is demethylated, coupled with extensive local non-CG hypermethylation of small interfering RNA–targeted sequences. Mutation of DEMETER partially restored endosperm CG methylation to levels found in other tissues, indicating that CG demethylation is specific to maternal sequences. Endosperm demethylation is accompanied by CHH hypermethylation of embryo transposable elements. These findings demonstrate extensive reconfiguration of the endosperm methylation landscape that likely reinforces transposon silencing in the embryo. This work culminated in the following publication where I was a co-first author: *Hsieh, T.-F., Ibarra, C.A., Silva, P., Zemach, A., Eshed-Williams, L., Fischer, R.L., Zilberman, D. (2009) Genome-wide Demethylation of Arabidopsis Endosperm. Science. 324:1451-1454.* The experiments described in this NIH predoctoral research proposal represent how I intend to follow up on these discoveries and complete my dissertation research.