PI: **Faubion, William A**	Title: Inflammatory cascades disrupt Treg function through epigenetic mechanisms	
Received: 07/02/2015	FOA: PA13-302	Council: 01/2016
Competition ID: FORMS-C	FOA Title: RESEARCH PROJECT GRANT (PARENT R01)	
2 R01 AI089714-06A1	Dual: DK	Accession Number: 3841834
IPF: 4976101	Organization: MAYO CLINIC ROCHESTER	
Former Number:	Department: Internal Medicine	
IRG/SRG: GMPB	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 6: [redacted] Year 7: [redacted] Year 8: [redacted] Year 9: [redacted] Year 10: [redacted]	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Stephen Ekker PhD	Mayo Clinic	Other (Specify)-Other Significant Contributor
Thomas Smyrk MD	Mayo Clinic	Other (Specify)-Other Significant Contributor
Raul Urrutia MD	Mayo Clinic	Other (Specify)-Other Significant Contributor
William Faubion MD	Mayo Clinic	PD/PI
Chandrashekhar Pasare PhD	University of Texas Southwestern Medical Center at Dallas	Consultant

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OMB Number: 4040-0001
Expiration Date: 06/30/2016

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE | **State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier [redacted]

b. Agency Routing Number

2. DATE SUBMITTED | **Application Identifier** | **c. Previous Grants.gov Tracking Number**

5. APPLICANT INFORMATION **Organizational DUNS*:** [redacted]

Legal Name*: Mayo Clinic
Department: Internal Medicine
Division: Gastroenterology
Street1*: 200 First Street SW
Street2:
City*: Rochester
County:
State*: MN: Minnesota
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 559050001

Person to be contacted on matters involving this application
Prefix: First Name*: David Middle Name: M Last Name*: Moertel Suffix:
Position/Title: Institutional Official
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: [redacted] Email: [redacted]

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

7. TYPE OF APPLICANT* M: Nonprofit with 501C3 IRS Status (Other than 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 Institute of Allergy and Infectious Diseases

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Inflammatory cascades disrupt Treg function through epigenetic mechanisms

12. PROPOSED PROJECT
Start Date* Ending Date*
07/01/2016 06/30/2021

13. CONGRESSIONAL DISTRICTS OF APPLICANT
MN-001

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: William Middle Name: A[redacted] Last Name*: Faubion Suffix: MD

Position/Title: Associate Professor
Organization Name*: Mayo Clinic
Department: Internal Medicine
Division: Gastroenterology
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$[redacted]
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$[redacted]
d. Estimated Program Income*	$0.00

16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ❍ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

❍ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: David Middle Name: M Last Name*: Moertel Suffix:

Position/Title*: Institutional Official
Organization Name*: Mayo Clinic
Department: RS-Research Services
Division: RS-Research Services
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted] STATES
ZIP / Postal Code*: [redacted]
Phone Number*[redacted] Fax Number: [redacted] Email*: [redacted]

Signature of Authorized Representative*
David.Moertel

Date Signed*
07/02/2015

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:

424 R&R and PHS-398 Specific
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OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Mayo Clinic
Duns Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: MN-001

File Name

Additional Location(s)

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: 1 2 3 4 5 6 If

NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number A3291-01

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	Project Summary_Abstract.pdf
8. Project Narrative*	Project_Narrative.pdf
9. Bibliography & References Cited	Final_bibliography.pdf
10.Facilities & Other Resources	Facilities_and_Other_Resources.pdf
11.Equipment	Equipment.pdf

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PROJECT SUMMARY/ABSTRACT:

The transcription factor FOXP3 is critical to the regulation of numerous debilitating human immune-mediated diseases. Very recently, the essential role for the histone methyltransferase (HMT) EZH2 in the epigenetic regulation and function of FOXP3 has been described. Inflammatory pathways modify EZH2 activity, and inflammatory signaling impairs Treg function *in vivo* and *in vitro*. The biological impact of the FOXP3-EZH2 pathway to IBD is unknown. Our *long-term goal* is to dissect epigenetic mechanisms regulating Treg cellular differentiation and function, particularly within the setting of GI inflammatory diseases. These discoveries will facilitate design of human cell therapy trials for IBD. The *objective* of this grant is to characterize the role for EZH2 in Treg suppressive function. The *central hypothesis* is that EZH2 plays a critical role in the homeostasis of Treg cells, and the disruption of EZH2 function by inflammatory signaling pathways contributes to IBD. Our *rationale* is that identification of the mechanism(s) to restore Treg suppressive function in the setting of intestinal inflammation will offer new therapeutic opportunities. Our *specific aims* will test the following hypotheses: (Aim1) Repression of immunoregulatory gene networks by FOXP3 requires the formation of a complex between this transcription factor and EZH2; (Aim 2) Inflammatory stimuli, such as IL6 lead to EZH2 phosphorylation and thereby disrupt the enzymatic activity of this epigenomic regulator; (Aim 3) Inhibition of the IL6 to EZH2 signaling pathway permits sustained Treg suppressive function in the setting of intestinal inflammation. Upon conclusion, we will understand the role for EZH2 in Treg loss of function in the setting of active inflammation. *This contribution is significant* since it will establish that several pathways targeted by available therapies (ie IL1β, IL6, TNFα) have the potential to regulate EZH2 HMT activity through post-translational modifications. Furthermore, current Treg cell therapy trials, while promising have not addressed the key issue of *in vivo* inflammation-induced disruption of Treg function. *The proposed research is innovative* because we investigate the effect of inflammatory signaling pathways on epigenetic complexes in Treg cells, a heretofore-unexamined process. Insight into epigenetic mechanisms is impactful as T cell progenitor cells inherit the parent transcriptional profile and unlike genetic change, they are modifiable by currently available therapy.

PROJECT NARRATIVE:

The proposed research is relevant to the public health because IBD, increasing in prevalence, represents a major national cost measured by both patient suffering and economic burden; and despite significant advances in care, clinical trial data demonstrate remission rates at best of 40%. Upon conclusion, we will understand the role for EZH2 in Treg loss of function in the setting of active inflammation, and this discovery will stimulate the opening of a new avenue in therapeutics directed at stimulation of autologous Treg cells to function within the inflammatory milieu.

FACILITIES AND OTHER RESOURCES:

Mayo Clinic's Research Resource/Core Service Facilities

Resource	Director
Medical Genome Facility *Includes the following Resources/Cores*: • *Biospecimens Accessioning Processing (BAP)* • *Pathology Research Core* • *Gene Expression Core* • *Cytogenetics Shared Resource (CSR)* • *Genotyping Shared Resource Core Facility* • *Sequencing Core*	Eric D. Wieben, Ph.D. • *W. Edward Highsmith, Jr., Ph.D. & Mine Cicek, Ph.D.* • *Thomas Flotte MD.* • *Jin Jen MD, PhD.* • *Patricia Greipp, DO.* • *Julie Cunningham, Ph.D.* • *Eric D. Wieben, Ph.D.*
Analytical Nuclear Magnetic Resonance (NMR)	Slobodan I. Macura, Ph.D.
Mayo Antibody Core Facility Rochester (MACFR)	Mr. Thomas G. Beito
Biomathematics Resource	Zeljko Bajzer, Ph.D. & Armando Manduca, Ph.D.
Biomedical Imaging Resource	Richard A. Robb, Ph.D.
Electron Microscopy Resource	Jeffrey L. Salisbury, Ph.D.
Flow Cytometry/Optical Morphology Resource	Richard G. Vile, Ph.D.
Gene Targeted Mouse Core Facility (GTM-CF)	Jan van Deursen, Ph.D.
Immunochemical Laboratory Core Facility	Ravinder J. Singh, Ph.D.; Joseph P. McConnell, Ph.D.; & George G. Klee, M.D., Ph.D.
Materials and Structural Testing Resource	Kai-Nan An, Ph.D. & Kenton R. Kaufman, Ph.D.
Mayo Clinic Cancer Center: *Includes the following Resources/Cores*: • *Biostatistics* • *Cancer Informatics* • *Clinical Research Office* • *Gene and Virus Therapy* • *Immunotherapy* • *Pharmacology* • Pharmacy • Survey Research	Robert B. Diasio, M.D. • *Daniel J. Sargent, Ph.D. & Vernon S. Pankratz, Ph.D.* • *James R. Cerhan, M.D. & Christopher G. Chute, M.D.,* • *Steven R. Alberts, M.D. & Janet Olson, Ph.D.* • *Mark J. Federspiel, Ph.D.* • *Dennis A. Gastineau, M.D.; Eugene Kwon, M.D.; & Stanimir Vuk-Pavlovic, Ph.D.* • *Matthew M. Ames, Ph.D.* • *Mr. Darryl C. Grendahl* • *Timothy J. Beebe, Ph.D.*
Mayo Proteomics Research Center (MPRC)	Daniel J. McCormick, Ph.D.

EQUIPMENT:

The PI's laboratory is equipped with most items for modern biochemistry, cell and molecular biology, and cellular immunology including 5 conventional PCR machines, 2 real time fluorescent PCR machines, a Phospholmager, 2 HPLC, FPLC, a spectrophotometer, electroporator, nucleofection unit, an ultracentrifuge, a mid-speed centrifuge and rotors, sonicator, scintillation counter, -20°C and -80°C freezers, a speed vac concentrator and a slab gel dryer. A Zeiss fluorescence confocal microscope is also available, fully equipped with an Eppendorf microinjection system for semi-automatic injection into single cells, along with an Icyte® Imaging Cytometer. There is also a cell biology and tissue processing laboratory, which has a biohood, cell incubators, centrifuge, refrigeration equipment, and purified water supplies. A computational biology laboratory with 5 Silicon Graphic Stations and 50 parallel CPU is used to model mutations. Biophysical equipment include for circular dicroism and isothermal titration calorimetry. Two fully equipped tissue culture facilities with 2 hoods each and a total of 12 incubators. The GI unit also has a fully equipped Becton Dickinson LSR II Fluorescent Activated Cell Sorter (FACS) on the floor. Kodak X-omat X-ray film processor and dark room are located on the floor.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: William Middle Name A[redacted] Last Name*: Faubion Suffix: MD

Position/Title*: Associate Professor
Organization Name*: Mayo Clinic
Department: Internal Medicine
Division: Gastroenterology
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*:
Zip / Postal Code*:

Phone Number*[redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: MD Degree Year:

	File Name
Attach Biographical Sketch*:	Biosketch_Faubion_6__26__2015.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302 . Received Date: 2015-07-02T14:04:51.000-04:00

PROFILE - Senior/Key Person

Prefix: First Name*: Chandrashekhar Middle Name Last Name*: Pasare Suffix: PhD

Position/Title*: Associate Professor
Organization Name*: University of Texas Southwestern Medical Center at Dallas
Department:
Division:
Street1*: [redacted]
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City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]

Phone Number*[redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Consultant Other Project Role Category:

Degree Type: PhD Degree Year:

Attach Biographical Sketch*: File Name Biosketch_PasareJune_2015.pdf

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Stephen Middle Name C Last Name*: Ekker Suffix: PhD

Position/Title*: Professor
Organization Name*: Mayo Clinic
Department: Biochem & Molecular Biology
Division: Biochem & Molecular Biology
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]

Phone Number*[redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) Other Project Role Category: Other Significant Contributor

Degree Type: PhD Degree Year:

Attach Biographical Sketch*: File Name Ekker_biosketchJune_2015.pdf

Attach Current & Pending Support:

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302 . Received Date: 2015-07-02T14:04:51.000-04:00

PROFILE - Senior/Key Person

Prefix: First Name*: Thomas Middle Name C Last Name*: Smyrk Suffix: MD

Position/Title*: Professor
Organization Name*: Mayo Clinic
Department: DLMP
Division: Anatomic Pathology
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number* [redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) Other Project Role Category: Other Significant Contributor

Degree Type: MD Degree Year:

File Name
Attach Biographical Sketch*: Biosketch_SmyrkJune_2015.pdf

Attach Current & Pending Support:

PROFILE - Senior/Key Person

Prefix: First Name*: Raul Middle Name Alfredo Last Name*: Urrutia Suffix: MD

Position/Title*: Professor
Organization Name*: Mayo Clinic
Department: Internal Medicine
Division: Gastroenterology
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number* [redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) Other Project Role Category: Other Significant Contributor

Degree Type: MD Degree Year:

File Name
Attach Biographical Sketch*: Biosketch_UrrutiaJune_2015.pdf

Attach Current & Pending Support:

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302 . Received Date: 2015-07-02T14:04:51.000-04:00

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: **Faubion, William**

eRA COMMONS USER NAME: [redacted]

POSITION TITLE: Associate Professor of Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Dartmouth College	B.A.	1988	Geology
University of Texas at Houston Medical School	M.D.	1992	Medicine
University of Texas at Houston Medical School		1992-1996	Int Med/Pediatrics
Mayo Clinic, Rochester, Minnesota		1996-2001	Gastroenterology
Beth Israel, Harvard, MA		2001-2003	Mucosal Immunology

A. Personal Statement

I am an expert in both pediatric and adult inflammatory bowel disease with a laboratory-based research program on the mucosal immunology of chronic intestinal inflammation. My current NIH funding supports research relevant to the developmental pathways towards T-regulatory cell development. Specifically, we study coupling of KLF family transcription factor binding to chromatin modifying complexes leading to epigenetic change in gene networks regulating T cell phenotypes. We have published key epigenetic mechanisms regulating stability of FOXP3 gene transduction and T regulatory cell development. We correlate biophysical and biochemical mechanisms to function using animal models of colitis. I have multiple models in the lab of chronic intestinal inflammation which serve to dissect molecular mechanisms of T-cell phenotypes related to chronic inflammation. I am a joint member in the Department of Immunology, and hold the rank of Associate Professor of Immunology (in addition to Medicine and Pediatrics), a rank at Mayo Clinic given upon merit of accomplishments specifically within the field of Immunology. In addition to this ongoing NIH-funded, laboratory-based research program, I am a well-recognized expert in clinical inflammatory bowel disease. I am the director of the Pediatric Inflammatory Bowel Disease Clinic and co-investigator in the adult IBD Clinic, where we collectively see over 4000 patients per year.

B. Positions and Honors

Positions and Employment

1992-1996 University of Texas at Houston, Internal Medicine/Pediatrics Residency
1997-1998 Mayo Clinic, Rochester, MN, Center for Basic Research in Digestive Diseases, Gastrointestinal Fellow, Laboratory of Gregory J. Gores, M.D.
1996-2000 Mayo Clinic, Pediatric and Adult Gastroenterology, Fellowship
2000-2003 Instructor of Medicine, Mayo Medical School
2001-2006 Mayo Foundation Scholar

2001-2003 Instructor of Medicine, Division of Immunology, BIDMC, Harvard Medical School
2003-2012 Assistant Professor of Medicine, Mayo Clinic College of Medicine
2012-present Associate Professor of Medicine, Mayo Clinic College of Medicine
2012-present Associate Professor of Pediatrics, Mayo Clinic College of Medicine
2013-present Associate Professor of Immunology, Mayo Clinic College of Medicine
2013-present Director, T32 Training Grant
2014-present Research Chair, Division of Gastroenterology and Hepatology, Mayo Clinic

Other Experience and Professional Memberships

2007 -2013 Member, American Board of Pediatrics
2007-present Associate Editor, Inflammatory Bowel Disease Journal
2007-present Associate Editor, Pediatric Inflammatory Bowel Disease
Member, American Board of Internal Medicine
2011-present Member, American Gastroenterological Association
2011-present Member, American College of Gastroenterology
Member, North American Society for Pediatric Gastroenterology and Nutrition
2011-present NIH Study Section Reviewer: Digestive Diseases and Nutrition
2012-present CCFA Career Development & Research Fellowship Committee Member
2015-2018 CCFA Research Fellowship Awards Committee Member

Honors

2000 J. Arnold Bargen Award – for outstanding achievement by a fellow in gastroenterology, Mayo Clinic
2005 REGAL (Research Excellence in GI and Liver)–for third year fellows or junior faculty members who are less than five years out of their fellowship or residency in GI who demonstrate the ability to conduct important research in the areas of upper GI, lower GI, outcomes, or hepatobiliary research
2005 Berry Family Foundation Scholar Award–to support Mayo GI staff who pursue educational opportunity at other institutions and then return to Mayo to set up their own research

C. Contribution to Science

1. The SLAM family regulates colitis through both adaptive and innate immune mechanisms.
Upon joining the Terhorst laboratory in 2001, the SLAM family of immune receptors was known to be relevant to lymphoproliferative syndromes however precise mechanisms of immunoregulation were unknown. As part of this collaborative team, we defined the role for SLAM, CD48, and Ly108 in T cell and innate immune cellular function in colitis. As SLAM is a measles virus receptor, the major impact of our work on SLAM as a regulator of phagosome function is on the field of virology and retargeting of measles virus.
   a. Wang, N. Satoskar, A., Faubion, W.A., Howie, D., Okamoto, S., Feske, S., Gullo, C., Clark, K., Sosa, M.R., Sharpe, A.H., Terhorst, C. (2004). The cell surface receptor SLAM controls T cell and macrophage functions. The Journal of Experimental Medicine, 199(9):1255-64. PMCID:2211908.
   b. Howie, D., Laroux, F.S., Morra, M., Satoskar, A.R., Rosas, L.E., Faubion, W.A., Julien, A., Rietdijk, S., Coyle, A.J., Fraser, C., Terhorst, C. (2005). Cutting edge: the SLAM family receptor Ly108 controls T cell and neutrophil functions. Journal of Immunology, 174(10):5931-5. PMID:15879084.
   c. Abadia-Molina, A.C., Ji, H., Faubion, W.A., Julien, A., Latchman, Y., Yagita, H., Sharpe, A., Bhan, A.K., Terhorst, C. (2006). CD48 controls T-cell and antigen-presenting cell functions in experimental colitis. Gastroenterology, 130(2):424-34. PMID:16472597.
   d. Berger, S.B., Romero, X., Ma, C., Wang, G., Faubion, W.A., Liao, G., Compeer, E., Keszei, M., Rameh, L., Wang, N., Boes, M., Regueiro, J.R., Reinecker, H.C., Terhorst, C. (2010). SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. Nature Immunology, 11(10):920-7. PMCID: PMC3338319.

2. Traditional innate immune receptors regulate function of human FOXP3+ Treg cells.

The FOXP3+ Treg cell was defined during the time frame of my post-doctoral training with the Terhorst laboratory, and we were the first to characterize the effect of colitis on Treg thymic developmental steps. Upon my return to Mayo clinic, we continued on with Treg related research with a particular focus on human physiology. With unique experience in innate immune receptors and Treg biology, we were the first to demonstrate a role for both cell membrane (TLR10) and intracellular (NOD2) pathogen recognition receptors on human Treg cellular function. In particular, the work on TLR10 and FOXP3 led to our subsequent deeper line of investigation on FOXP3 gene transcriptional events.

a. Faubion, W.A., De Jong, Y.P., Molina, A.A., Ji, H., Clarke, K., Wang, B., Mizoguchi, E., Siimpson, S.J., Bhan, A.K., Terhorst, C. (2004). Colitis is associated with thymic destruction attenuating CD4+25+ regulatory T cells in the periphery. Gastroenterology, 126(7):1759-70.
b. Abadia-Molina, A.C., Mizoguchi, A., Faubion, W.A., De Jong, Y.P., Rietdjik, S.T., Comiskey, M., Clarke, K., Bhan, A.K., Terhorst, C. (2005). In vivo generation of oligoclonal colitic CD4+ T-cell lines expressing a distinct T-cell receptor Vbeta. Gastroenterology, 128(5):1268-77.
c. Bell, M.P., Svingen, P.A., Rahman, M.K., Xiong, Y., Faubion, W. A. Jr. (2007) FOXP3regulates TLR10 expression in human T regulatory cells. Journal of Immunology, 173(3):1893-900. PMID:17641056.
d. Rahman, M.K., Midtling, E.H., Svingen, P.A., Xiong, Y., Bell, M.P., Tung, J., Smyrk, T., Egan, L.J., Faubion Jr, W.A. (2010). The pathogen recognition receptor NOD2 regulates human FOXP3+ T cell survival. Journal of Immunology, 184(12):7247-56. PMCID: 38886856.

3. KLF family members regulate FOXP3 and Treg function through coupling to epigenetic complexes. Careful characterization of the FOXP3 core promoter led to the earliest recognition of epigenetic mechanisms leading to FOXP3 gene activation and Treg development. Our work on the coupling of KLF transcription factors to chromatin modifying complexes has advanced the understanding of Treg biology, CD8+ T cell function, and intestinal stem cell function. The greatest impact has been the first recognition of the role for the histone methyltransferase EZH2 in Treg biology.

a. Xiong, Y., Khanna, S., Grzenda, A.L., Sarmento, O.F., Svingen, P.A., Lomberk, G.A., Urrutia, R.A., Faubion Jr, W.A. (2012). Polycomb antagonizes p300/CREB-binding protein-associated factor to silence FOXP3 in a Kruppel-like factor-dependent manner. Journal of Biological Chemistry, 287(41):34372-85. PMCID: PMC3464543.
b. Xiong, Y., Svingen, P.A., Sarmento, O.F., Smyrk, T.C., Dave, M., Khanna, S., Lomberk, G.A., Urrutia, R.A., Faubion Jr, W.A. (2014). Differential coupling of KLF10 to Sin3-HDAC and PCAF regulates the inducibility of the FOXP3 gene. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 307(6)R608-20. PMCID: PMC4166759.
c. Sarmento, O.F., Svingen, P.A., Xiong, Y., Xavier, R.J., McGovern, D., Smyrk, T.C., Papadakis, K.A., Urrutia, R.A., Faubion, W.A. (2015). A novel role for KLF14 in T regulatory cell differentiation. Cellular and Molecular Gastroenterology and Hepatology, 1(2):188-202. PMCID: PMC4349492.
d. Papadakis, K.A., Krempski, J., Reiter, J., Svingen, P., Xiong, Y., Sarmento, O.F., Huseby, A., Johnson, A.J., Lomberk, G.A., Urrutia, R.A., Faubion, W.A. (2015). Kruppel-like factor KLF10 regulates transforming growth factor receptor II expression and TGF-beta signaling in CD8+ T lymphocytes. The American Journal of Physiology- Cell Physiology, 308(5):C362-71. PMCID: PMC4346734.
e. Dave, M., Hayashi, Y., Gajdos, G.B., Smyrk, T.C., Svingen, P.A., Kvasha, S.M., Lorincz, A., Dong, H., Faubion, W.A., Jr., Ordog, T. (2015). Stem cells for murine interstitial cells of cajal suppress cellular immunity and colitis via prostaglandin e2 secretion, 148(5):978-90. PMCID:PMC4409492.

A full list of research-related published work can be found at:
http://www.ncbi.nlm.nih.gov/sites/myncbi/william.faubion.1/bibliography/40757558/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

[redacted]

R01AI089714-02 Faubion (PI) 2/15/11 – 1/31/16
The goal of this study is to investigate transcriptional regulation of regulatory T cell (Treg) developed by Kruppel-like factor 10 (KLF10) and its role in immune homeostasis.

[redacted]

[redacted]

[redacted]

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Chandrashekhar Pasare

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Associate Professor of Immunology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Bidar Veterinary College, Bidar, India	B.V.Sc	06/1992	Veterinary Medicine
Indian Veterinary Research Institute, Bareilly, India	M.V.Sc	05/1994	Immunology and Microbiology
National Institute of Immunology, New Delhi, India	Ph.D.	09/2000	Immunology and Molecular Biology
Yale University School of Medicine, New Haven, CT	Postdoctoral	05/2006	Innate control of Adaptive Immunity

A. Personal Statement

I have been working in the field of innate immunity and innate control of adaptive immunity for about 14 years. In addition to a Ph.D. in Immunology, I have had post-doctoral training at Yale University, where I worked on understanding the importance of the TLR signaling pathway in activation of adaptive immune responses. This work led to understanding of TLR mediated regulation of T and B cell responses leading to publications in Science (1928 citations), Immunity (325 citations) and Nature (537 citations). My laboratory continues to work in the area of innate control of adaptive immunity and our more recent work has also focused on understanding molecular details of TLR signaling and molecular mechanisms by which the innate immune system regulates the adaptive immune responses. My lab has extensive experience in studying TLR mediated activation of adaptive immunity and our recent work has led to new understanding of regulation of immune responses in the gut (ref # 2a), discovery of a new TLR signaling adapter that links TLRs to PI3K activation (ref # 3a) and discovery of a rapid NLRP3 inflammasome activation pathway controlled by IRAK-1 (ref # 4a). We have also recently demonstrated that plasma membrane and surface TLRs have different functions in regulation of CD8 T cell responses against pathogens (ref # 1c).

B. Positions and Honors

Positions and Employment

2000-2005	Post-doctoral Associate, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT
2005-2006	Associate Research Scientist, Section of Immunobiology, Yale University School of Medicine, New Haven, CT
2006-2007	Scientist, Immunology, Genentech, Inc. South San Francisco, CA
2007-2014	Assistant Professor, Department of Immunology, The University of Texas Southwestern Medical Center, School of Medicine, Dallas, TX
2014-present	Associate Professor, Department of Immunology, The University of Texas Southwestern Medical Center, School of Medicine, Dallas, TX

Professional Memberships

1999	Life member, Indian Immunology Society
2010 -	Member, American Association of Immunologists

Honors/Awards

1992-1994	Indian Council of Agricultural Research's Junior Research Fellowship
1994-1999	Senior Research Fellowship from Department of Biotechnology, India
2000-2005	Howard Hughes Medical Institute, Post-Doctoral Fellowship
2007	UT Southwestern Medical Center Endowed Scholar in Biomedical Research
2013	AAI early career faculty travel award

C. Contribution to Science

1. When I started my post-doctoral work in the laboratory of Ruslan Medzhitov about 14 years ago, TLRs were only just discovered and not much was known about the various mechanisms by which TLRs influence adaptive immunity. In addition there was renewed interest in a population of cells called regulatory T cells or suppressor T cells. These cells, characterized by surface expression of CD4 and CD25 markers, were shown to be potent suppressors of naïve T cell activation. The general consensus was that these cells block activation of auto-reactive cells. A key question for us was: How do pathogen specific T cells over come the block induced by regulatory T cells to mount protective immune responses? We hypothesized that, since TLRs are major sensors of infections, TLR activation on DCs should be able to overcome suppression mediated by regulatory T cells (Treg cells). I used TLR-deficient and TLR-sufficient dendritic cells for in vitro suppression assays to show that TLR activation on DCs overcomes the block induced by regulatory T cells. In addition I showed that surface maturation of DCs is not sufficient to overcome Treg cell mediated suppression. Finally I demonstrated that the cytokine IL-6 secreted by DCs in response to TLR ligands is responsible for providing critical signals necessary for naïve T cells to overcome Treg cell mediates suppression. In summary, I discovered that the Toll-pathway of DC activation controls adaptive immune responses by at least two major mechanisms; 1. By induction of surface maturation of DCs and 2: By inducing secretion of IL-6, which overcomes Treg cell mediated suppression. This study was described as ground breaking in a commentary that accompanied the published article in the journal "Science". After gaining insights into the critical requirements for TLR activation on DCs in inducing activation of naïve T cells in vitro, I set out to understand the in vivo significance of these findings and to understand the dynamic interactions between innate and adaptive immune systems in a living animal. Immunologists over the years have been using protein antigens to study induction of T and B cell responses in vivo. I showed using pure protein preparations that, naïve T cells could not be activated in vivo if immunized in the absence of a TLR ligand. Most commercial protein preparations are contaminated with TLR ligands and hence induce T and B cell activation when immunized in depot forming adjuvant such as alum. I used MyD88-deficient mice to shown the importance of different aspects of DC activation for T cell priming in vivo. I showed that DCs mature and migrate to the draining lymph node in response to LPS in MyD88-deficient mice but fail to induce naïve T cell activation. This was very contrary to the current understanding that expression of MHC and co-stimulatory molecules is sufficient to prime antigen specific T cells. I showed that this is not the case and that Treg cells block activation of naïve T cells if DCs do not secrete cytokines during priming. I was thus able to define the critical requirements for naïve T cell activation. Further, I discovered that TLR and MyD88 dependent signals are necessary for induction of memory CD4 T cells. This is an important finding and there is ongoing research in my current lab to understand the cellular and molecular mechanisms of how TLR activation induces development of CD4 memory T cells. In a related study dissecting the role of TLRs in regulating B cell responses I discovered that the poor antibody responses in MyD88 deficient mice were because of lack of TLR signaling in B cells rather than lack of T cell priming and differentiation. This study for the first time established the role of innate immune sensing in mounting T-dependent B cell responses and implicated B cell intrinsic TLR signaling in B cell activation and differentiation. I was a post-doctoral fellow for all these studies.

a. **Pasare, C.,** and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. **Science** *299*, 1033-1036. PMID: 12532024.

b. **Pasare, C.,** and Medzhitov, R. (2004). Toll-dependent control mechanisms of CD4 T cell activation. **Immunity** *21*, 733-741. PMID: 15539158.

c. **Pasare, C.,** and Medzhitov, R. (2005). Control of B-cell responses by Toll-like receptors. **Nature** *438*, 364-368. PMID: 16292312.

2. As an independent investigator I continued to focus on understanding the role of innate immune system in regulating adaptive immunity. In the first study published from the laboratory we found that the cytokine requirements for induction of the inflammatory Th17 lineage cells depend on the site of priming. We found that the mucosal and systemic immune systems have different rules for inducing differentiation of naïve T cells into Th17 cells. We discovered that IL-1R mediated, MyD88 dependent signaling in CD4 T cells is critical for generation of Th17 lineage cells in all lymphoid tissues. However, while there is a requirement for IL-6 for Th17 priming in the mucosal tissues such as the lamina propria of the intestines as well as the lungs, IL-6 is not required for generation of Th17 lineage cells in the peripheral lymphoid organs such as the spleen and lymph nodes, The differential requirement for Th17 priming in the peripheral lymphoid organs versus the lamina propria of the intestines is true both during steady state as well as during exposure to pathogens via oral or systemic route. We also demonstrate that lack of IL-6 leads to higher proportion of Foxp3 positive CD4 T cells selectively only in the lamina propria of the intestines. Finally we demonstrate that gut specific need for IL-6 is dictated by DCs resident in the lamina propria of the intestines as LP DCs from IL-6 deficient mice fail to prime Th17 lineage cells. DCs resident in the spleens of IL-6 deficient mice induce normal priming of Th17 lineage cells. This is the first demonstration of selective need of different cytokines based on priming micro-environments for any kind of helper T cell lineage. Our study challenges the concept that IL-6 is a master regulator of RORgt and Th17 differentiation and provides novel insights into tissue specific requirements for T cell differentiation. Differential requirement for Th17 priming in spleen and gut lamina propria is a novel concept and has important implications for targeting systemic and mucosal tissue specific auto-immunity as well as for determining routes of vaccination. In a related study that deals with regulation of CD8 T cell responses by the innate immune system, we found that plasma membrane TLRs and endosomal TLRs have a differential role in inducing CD8 T cell priming and this is primarily dictated by population of DCs recruited to the site of priming

   a. Hu, W., Troutman, T.D., Edukulla, R., and **Pasare, C.** (2011). Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. **Immunity** *35*, 1010-1022. PMID: 22137454. PMCID: PMC3246047.

   b. Hu, W and **Pasare, C** (2013). Location, location, location: tissue specific regulation of immune responses. **Journal of Leukocyte Biology.** *94,* 409-421. PMID:23825388. PMCID: PMC3747123.

   c. Mandraju R, Murray S, Forman J and **Pasare C**. (2014). Differential ability of surface and endosomal TLRs to induce CD8 T cell responses in vivo. **J Immunol.** *192*, 4303-15. PMID: 24688022. PMCID: PMC4002505.

3. TIR domains are required for the initiation of TLR signaling and serve to link TLRs to their adaptors through homotypic interactions. The known adapters of TLR signaling MyD88 (and TIRAP) and TRIF (and TRAM) signal to activate NF-kB, MAP kinases and IRF proteins but it has never been clear how TLR signaling leads to PI3 kinase activation. Although NF-kB activation is important for inducing pro-inflammatory response and cytokine secretion by macrophages, PI3K activation in cells plays a critical role in regulating cell survival, cell cycling and proliferation. We have found that BCAP, via its TIR domain, links TLRs to PI3K activation, and is also responsible for negatively regulating TLR signaling. Our studies demonstrate that BCAP modulates pro-inflammatory responses and BCAP deficient mice have exaggerated inflammation in response to infections and are highly susceptible to colitis. The study of TLR mediated activation of NF-kB has yielded many important and seminal discoveries into the regulation of this inflammatory pathway. However, signaling via TLRs to PI3K is poorly characterized. Data using chemical inhibitors suggests contradictory roles for PI3K. Due to the central and important role of PI3K through many

converging signaling pathways, genetic tools utilizing mice deficient for PI3K components carry the caveat of disrupted signaling through other signaling pathways. Our delineation of BCAP's critical role in activation of the PI3K axis is important not only through our description of how BCAP regulates inflammation, but also because of the characterization of a signaling adapter critically required for the proximal bridging of TLRs to PI3K.

a. Troutman, T.D., Hu, W., Fulenchek, S., Yamazaki, T., Kurosaki, T., Bazan, J.F., and **Pasare, C.** (2012). Role for B-cell adapter for PI3K (BCAP) as a signaling adapter linking Toll-like receptors (TLRs) to serine/threonine kinases PI3K/Akt. **Proc Natl Acad Sci USA** *109*, 273-278. PMID: 22187460. PMCID: PMC3252926.

b. Troutman, T.D., Bazan, J.F., and **Pasare, C.** (2012). Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. **Cell Cycle** *11*. PMID: 22895011. PMCID: PMC3478307.

4. In the past few years, my laboratory also got interested in understanding the biogenesis of IL-1beta. The cytokines IL-1beta and IL-18 are made as pro-forms and are induced by TLR signaling. When a cell senses a toxin or virulence factor or is undergoing stress, this leads to activation of inflammasome causing caspase-1 activation, which then cleaves IL-1beta and IL-18 for their release. The scientific understanding was that there is a priming signal (TLR activation) and an activation signal (NLR activation) separated by time, necessary for inflammasome activation. We hypothesized that when there is an infection by a virulent pathogen, the cells are likely to see both of these signals at the same time and that macrophages should be equipped to induce inflammasome activation independent of priming. Consistent with this hypothesis, we discovered a rapid NLRP3 inflammasome activation pathway that leads to caspase-1 activation in as little as 20 minutes following simultaneous sensing of TLR and NLRP3 ligands. More importantly we found that this activation is directly regulated by TLR signaling component IRAK-1 and that not only does IRAK-1 form part of the inflammasome complex, but its kinase activity is important for rapid NLRP3 inflammasome assembly. We also discovered that this rapid IRAK-1 dependent inflammasome activation pathway is critical to sense *Listeria monocytogenes* and induce rapid IL-18 dependent IFN-gamma production by NK cells and memory CD8 T cells. We believe that this rapid inflammasome activation pathway is critical for rapid innate defense and are continuing to work on understanding the molecular mechanism of this activation and its role in protection against a variety of virulent pathogens.

a. Lin KM, Hu W, Troutman, TD, Jennings M, Brewer, T, Li, X, Nanda, S, Cohen P, Thomas J and **Pasare C**. (2014) IRAK-1 by passes priming and directly links TLRs to rapid NLRP3 inflammasome activation. **Proc. Natl. Acad. Sci. USA.** *111*, 775-780. PMID: 24379360. PMCID: PMC3896167.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/1pmWT2zpU9bQM/bibliography/46106384/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

2015/04/15-2017/03/31
R21 AI115420, National Institute of Allergy and Infectious Diseases (NIAID, NIH)
PASARE, CHANDRASHEKHAR (PI)
Role of Microenvironmental cues in CD8 T cell activation and memory generation
The major goals of this project are to investigate the mechanisms by which priming microenvironments influence activation and development of CD8 memory T cells.

[redacted]

2010/08/15-2015/07/31
R01 AI082265, National Institute of Allergy and Infectious Diseases (NIAID, NIH)
PASARE, CHANDRASHEKHAR (PI)
Toll-like receptor mediated regulation of effector and memory CD4 T cell responses
The major goals of this project are to investigate the mechanisms by which TLRs influence activation and differentiation of CD4 T cells into effector and memory cells.

Competed Research Support (ended in the last 3 years)

2010/02/15-2013/01/31
R43 AI089138, National Institute of Allergy and Infectious Diseases (NIAID, NIH)
PASARE, CHANDRASHEKHAR (PI)
Synergistic vaccine adjuvants that stimulate both innate and adaptive immunity
The major goals of this project were to develop a glycoside adjuvant that can activate innate immunity and investigate its ability to induce protective adaptive immune responses to various pathogens.
Role: Principal Investigator

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

NAME: Stephen C. Ekker

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor of Biochemistry and Molecular Biology

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Illinois Urbana-Champaign, Urbana	B.S.	05/1988	Electrical Engineering
University of Illinois Urbana-Champaign, Urbana	B.S.	05/1988	Genetics & Dev. Biology
Johns Hopkins University, Baltimore, MD	Ph.D.	05/1993	Molec. Bio. & Genetics
Johns Hopkins University, Baltimore, MD	PostDoctoral	10/1995	Develop. Genetics

A. Personal Statement

My background in molecular genetics and genomics started with undergraduate research with a project to restriction map the genome of the Archaebacterium *Sulfolobus solfataricus*. I have been helping further the zebrafish (*Danio rerio*) as a model vertebrate to address major issues in human health and biology since training as a post-doctoral fellow. This system has the potential for diverse genetic and behavioral studies typically restricted to the world of the fly or worm but conducted within the biological framework of a vertebrate.

The major focus of my laboratory is on understanding our genome, using the zebrafish as a rapid molecular test system. We established the rapid use of morpholino sequence-specific knockdown technology for vertebrate functional genomics applications using the zebrafish as the pioneering model system fifteen years ago. In parallel, we developed vertebrate transposon tools including our protein trap gene-breaking vectors to generate a 700+ line collection of molecularly characterized and revertible mutant zebrafish lines, the first engineered conditional alleles in any organism outside the mouse. We have deployed transposons in diverse application areas including human T cells, zebrafish, and mice. Custom restriction endonucleases offer a third leg, targeted modification using genome editing tools. We continue to develop science behind these new engineering toolkits, working with an array of laboratories as diverse as rat, pig, mouse, nematode, and fly biologists in addition to regular colleagues that work in human cells and zebrafish.

As Director of the Genetics and Model Systems Core for the Center for Cell Signaling in Gastroenterology, I am delighted to support your project.

B. Positions and Honors

1986-1988	Undergraduate Research: Restriction Mapping the Genome of the Archaebacterium *Sulfolobus solfataricus.* University of Illinois; C.R. Woese, advisor
1988-1993	Graduate Dissertation Differential DNA Binding and the Specificity of Homeotic Gene Action, Philip A. Beachy, Advisor, Johns Hopkins School of Medicine (SOM) and HHMI, Baltimore, MD
1990-1992	Teaching Assistant, Dept. of Mol. Biol. and Genetics, Johns Hopkins University, Baltimore, MD
1990-1993	March of Dimes Birth Defects Predoctoral Fellow
1993-1995	Postdoc, Biochemical Properties and Biological Activities of the Hedgehog Gene Family, P.A. Beachy, Advisor, R.T. Moon, Co-Advisor, Johns Hopkins SOM and HHMI, Baltimore, MD
1995-2007	Assistant, Associate, and full Professor, University of Minnesota, Minneapolis, MN
1997-1999	March of Dimes Basil O'Connor Scholar
2000	Co-founder, Discovery Genomics, Inc.
1999-2007	Director, Arnold and Mabel Beckman Center for Transposon Research, University of Minnesota
2002-2007	Associate Head, Genetics, Cell Biology and Development, University of Minnesota
2007-present	Adjunct Professor, Genetics, Cell Biology and Development, University of Minnesota
2007-present	Professor Dept Biochemistry and Molecular Biology and Consultant, Mayo Clinic, Rochester,MN

2007-present Director, Mayo Clinic Zebrafish Facility
2007-present Mayo Predoctoral Education Programs Committees (CTS: 2007-present; BMB 2007-12)
2008-present Editor-in-Chief, The *Zebrafish* journal
2009-present Director, Genetics and Model Systems Core, Mayo Center for Cell Signaling in Gastroenterology
2010-present Director, Mayo Addiction Research Center
2012-present Founder, InSciEd Out Foundation
2014-present Chairman of the Board, InSciEd Out Foundation
2013-present Member, Faculty of 1000
2013 Co-Organizer and Founder, 1st International Zebrafish for Personalized/Precision Medicine Conference, Toronto, Oct. 16-18, 2013
2013-present Founding member, Zebrafish Disease Models Society
2014-present Director, Model Systems Core, Mayo Translational PKD Center

Professional Memberships
AAAS (1992), Genetics Society of America (1992), SDB (1996), ASHG (2005), SFN (2010), IBANGS (2013)
Honors (selected) Member, National Honor Society (1983); James Scholar and Dean's List, University of Illinois (1983-88); Graduation with Honors, University of Illinois (1988); Member, Phi Kappa Phi and Eta Kappa Nu Honor Societies (1988); Predoc fellow, March of Dimes (1990-93); Basil O'Connor Scholar, March of Dimes (1997-99) Major Study Panels: NSF Dev Mech (2002-2004); NCRR 10/99; NHLBI SEP 06/00; NIGMS PPG 01/02, CDF4 06/02; Craniofacial Special Emphasis Panel 11/02; NIDDK SEP 11/02; Genome 02/03, 12/03; Innov. Tox Models SEP 07/03; NDPR 10/03, 06/04; Tools for Genetic Studies in Zebrafish 04/04, 03/09; Centers for Biomedical Computing SEP, 05/05; NCI Cancer Nanotech Platform SEP (section chair) 07/05; NIH MDCN SEP 07/05; NIH CAD&O 10/05, 06/06, 10/06; NHLBI 03/06; SBIR 04/06; Neurogenesis and Cell Fate Full member, 06/05 – 06/09; RC4 05/10; NHLBI 11/10; Member of the NIH College of CSR Reviewers 01/2010-present; NIDA-K Full member 06/10-12 (end of panel); NIDA ZDA1 EXL-T Full member 06/10-present; NIH/NCATS Comparative Medicine (CM) Special Emphasis Panel (SEP) Feb 13-14, 2013; NIDA CEBRA Oct 2013, Mar 2014; NIH PPG review April 2014

Pertinent Science Education: served as Editor-in-Chief for two Special Issues of the Zebrafish on the topic 'Zebrafish in Education.' I solicited funding to cover all publication charges (including NIH support), making all of these issues freely available to classroom teachers and students around the world. The first issue came out in 2009, the second in December of 2012.

Patents
Ekker,S.C. et al. Inhibition of Gene Expression using Polynucleotide Analogues. US Patent 6,867,349.
Ekker,S.C. et al., Syndecans and Angiogenesis – US Patent 7,713,925.

C. Contributions to Science [125 publications; google h index 48]

1) Biochemical and biological activities of *hedgehog* signaling in *Drosophila* and vertebrates. During my post-doctoral training, I contributed to uncovering mechanistic understanding how this key pathway works in a variety of critical processes and in the cloning of vertebrate family members including the first work using zebrafish implicating *shh* signaling as defective in *holoprosencephaly*.
   a) Lee, J. J., **Ekker, S. C.,** von Kessler, D. P., Porter, J. A., Sun, B. I. and Beachy, P. A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528-1537.
   b) **Ekker, S .C.,** Ungar,A., Greenstein,P., von Kessler,D.P., Porter,J.A., Moon,R.T., and Beachy,P.A. (1995). Patterning Activities of Vertebrate hedgehog Proteins in the Developing Eye and Brain. *Current Biology*, 5: 944-955.
   c) **Ekker, S. C.,** McGrew, L. L., Lai, C. J., Lee, J. J., von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of Xenopus laevis. *Development* **121**, 2337-2347.
   d) Porter, J. A., **Ekker, S. C.,** Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V., et al. (1996). *Hedgehog* patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34.

2) Development of morpholino antisense technology for vertebrate genomics applications using the zebrafish.
   a) Nasevicius, A. and **Ekker, S. C.** (2000). Effective targeted gene 'knockdown' in zebrafish. *Nature genetics* **26**, 216-220.
   b) Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S. A. and **Ekker, S. C.** (2007). p53 activation by knockdown technologies. *PLoS genetics* **3**, e78.
   c) Bill, B. R., Petzold, A. M., Clark, K. J., Schimmenti, L. A. and **Ekker, S. C.** (2009). A primer for morpholino use in zebrafish. *Zebrafish* **6**, 69-77. PMCID: PMC2776066
   d) Patent awarded: **Ekker,S.C.** et al. Inhibition of Gene Expression using Polynucleotide Analogues. US Patent 6,867,349.

3) Development of transposons for vertebrate genomics applications in zebrafish. We deployed the first transposon system, *Sleeping Beauty,* for transgenesis and enhancer trapping, and then harnessed the endogenous *Tol2* transposon for conditional mutagenesis and protein trapping applications.
   a) Davidson, A. E., Balciunas, D., Mohn, D., Shaffer, J., Hermanson, S., Sivasubbu, S., Cliff, M. P., Hackett, P. B. and **Ekker, S. C.** (2003). Efficient gene delivery and gene expression in zebrafish using the *Sleeping Beauty* transposon. *Developmental biology* **263**, 191-202.
   b) Balciunas, D., Davidson, A. E., Sivasubbu, S., Hermanson, S. B., Welle, Z. and **Ekker, S. C.** (2004). Enhancer trapping in zebrafish using the *Sleeping Beauty* transposon. *BMC genomics* **5**, 62.
   c) Balciunas, D., Wangensteen, K. J., Wilber, A., Bell, J., Geurts, A., Sivasubbu, S., Wang, X., Hackett, P. B., Largaespada, D. A., McIvor, R. S., et al. **Ekker, S. C.** (2006). Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. *PLoS genetics* **2**, e169.
   d) Clark, K. J., Balciunas, D., Pogoda, H. M., Ding, Y., Westcot, S. E., Bedell, V. M., Greenwood, T. M., Urban, M. D., Skuster, K. J., Petzold, A. M., et al. **Ekker, S. C.** (2011). *In vivo* protein trapping produces a functional expression codex of the vertebrate proteome. *Nature methods* **8**, 506-515. PMCID: PMC3306164

4) *In vivo* genome editing. We developed new tools and approaches for genome editing including the first targeted knockins in zebrafish. This approach also works well in *in vitro* systems.
   a) Bedell, V. M., Wang, Y., Campbell, J. M., Poshusta, T. L., Starker, C. G., Krug, R. G., 2nd, Tan, W., Penheiter, S. G., Ma, A. C., Leung, A. Y., et al. **Ekker, S. C.** (2012). *In vivo* genome editing using a high-efficiency TALEN system. *Nature* **491**, 114-118. PMCID: PMC3491146
   b) Neff, K. L., Argue, D. P., Ma, A. C., Lee, H. B., Clark, K. J. and **Ekker, S. C.** (2013). Mojo Hand, a TALEN design tool for genome editing applications. *BMC bioinformatics* **14**, 1. PMCID: PMC3575288
   c) Ma, A. C., Lee, H. B., Clark, K. J. and **Ekker, S. C.** (2013). High efficiency *In vivo* genome engineering with a simplified 15-RVD GoldyTALEN design. *PloS ONE* **8**, e65259. PMCID: PMC3667041
   d) Peng, Y., Clark, K. J., Campbell, J. M., Panetta, M. R., Guo, Y. and **Ekker, S. C.** (2014). Making designer mutants in model organisms. *Development* **141**, 4042-4054. PMCID: PMC4302887

5) Establishment of Integrated Science Education Outreach (InSciEd Out), a new approach for excellence in science education for the betterment of human health for all (http://insciedout.org/). We are operational on three continents (North America, Africa and India). Our highly collaborative program focuses on core cultural change, implementing science education within the context and needs of each partner community.
   a) Pierret,C., Sonju,J., Leicester,J. Hoody,M., LaBounty,T., Frimannsdottir,K.,R., **Ekker,S.C.** (2012) Improvement in Student Science Proficiency Through InSciEd Out, *Zebrafish*, 9, 155-68. PMCID: PMC3529492
   b) Clark, K. J. & **Ekker, S. C.** (2015) How Zebrafish Genetics Informs Human Biology. *Nature Education* 8(4):3.

URL for bibliography:
http://www.ncbi.nlm.nih.gov/pubmed?Db=pubmed&Cmd=DetailsSearch&Term=ekker+sc%5BAll+Fields%5D&WebEnv=0SoUqK-GD_T4YuzbaQadqM8hNqScpHAFyiFvTbm7gSa2SDumUj4r3YHA2epX7E-HqWGGoT1pliY_npV%40255F54F3705678F0_0091SID&WebEnvRq=1

D. Research Support

Ongoing Research Support

Genome Biology

5R01/56-GM63904-11 (Ekker, PI; Farber, Hammerschmidt, Xu and Clark as Co-I) 09/01/01 – 08/31/16
"Systematic Vertebrate Functional Genomics"
This grant funds a transposon-based phenotypic screen in the zebrafish to assess the role of 500 genes in development, skin, heart and digestive system biology.

[redacted]

1R01 HG006431-04 (Ekker, PI) 08/01/11 – 07/30/17
"International Zebrafish Mutagenic Protein Trap"
This resource grant funds the generation of 1000 new gene-break transposon lines for the zebrafish community. There is no support for biological annotation in this grant.

Supporting Grants

1P30 DK84567-06 (PI: LaRusso, N/Genetics Core PI: Ekker) 09/01/09 – 08/31/19
"Mayo Center for Cell Signaling in Gastroenterology"
SCE is PI of the Genetics and Model Organism Core that is designed to bring consultation, training and state-of-the-art genetics tools and model organisms to local scientists.

P30 DK090728-5 (PI:Torres,V.E.; Ekker, Model Organism Core PI) 09/30/10 – 06/30/15
"Mayo Translational PKD Center (MTPC)"
Dr. Ekker is Director of the Model Systems Core. [The competitive renewal received a score of 11 and will likely be continued.]

Education Grants

2UL1TR000135-9 (PI: Khosla, S) 07/01/11 – 06/30/16
"Mayo Clinic Center for Translational Science Activities"
Dr. Ekker is Associate Director for the Predoctoral training program in translational sciences, including the K-8 InSciEd Out science education reform initiative for the healthy community program.

Completed Research Support

5R01 DA14546-10 (Ekker, PI) 05/01/01 – 06/30/14
"Intron-based Mutagenic Transposons for Zebrafish"
The grant funded the development of the gene-break transposon technology for zebrafish functional genomics including the use of GBTs for behavioral genetics of the nicotine response.

[redacted]

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Thomas C. Smyrk, M. D.

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor of Pathology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Minnesota, Minneapolis, MN	BA	07/78	English
University of Minnesota, Minneapolis, MN	MD	07/82	
Mayo Clinic, Rochester, MN		07/86	

Please refer to the Biographical Sketch sample in order to complete sections A, B, C, and D of the Biographical Sketch.

A. Personal Statement:

I have twenty-nine years of experience as a surgical pathologist with interest in gastrointestinal pathology. Colitis and colitis-associated dysplasia/carcinoma have been particular interests of mine. Dr. Faubion and I have successfully collaborated on several previous projects and I look forward to collaborating with him and his excellent colleagues in the near future.

1) Garrity-Park MM, Loftus EV, Bryant SC, Sandborn WJ, **Smyrk TC**. Tumor necrosis factor-alpha polymorphisms in ulcerative colitis-associated colorectal cancer. Am J Gastroenterol. 2008 Feb; 103(2):407-15. PMID:18289203. DOI:10.1111/j.1572-0241.2007.01572.x.
2)) Kisiel JB, Garrity-Park MM, Taylor WR, **Smyrk TC**, Ahlquist DA. Methylated eyes absent 4 (EYA4) gene promotor in non-neoplastic mucosa of ulcerative colitis patients with colorectal cancer: evidence for a field effect. Inflamm Bowel Dis. 2013 Sep;19(10):2079-83. PMID:23867875. PMCID:4119944. DOI:10.1097/MIB.0b013e31829b3f4d.
3) Sarmento OF, Svingen PA, Xiong Y, Xavier RJ, McGovern D, **Smyrk TC**, Papadakis KA, Urrutia RA, Faubion WA. A novel role for KLF14 in T regulatory cell differentiation. Cell Mol Gastroenterol Hepatol. 2015 Mar 1; 1(2):188-202.e4. PMID:25750932. PMCID:4349492. DOI:10.1016/j.jcmgh.2014.12.007.
4) Dave M, Hayashi Y, Gajdos GB, **Smyrk TC**, Svingen PA, Kvasha SM, Lorincz A, Dong H, Faubion WA Jr, Ordog T. Stem cells for murine interstitial cells of cajal suppress cellular immunity and colitis via prostaglandin e2 secretion. Gastroenterology. 2015 May;148(5):978-90. Epub 2015 Jan 28. PMID:25637652. PMCID:4409492.

B. Positions and Honors

Positions and Employment

1986-1990 Assistant Professor of Pathology, Creighton University, Omaha, NE
1990-1997 Associate Pathologist, Clarkson Hospital, Omaha, NE
1997-2000 Associate Professor of Pathology, University of Nebraska, Omaha, NE

2000-2012 Associate Professor of Pathology, Mayo Clinic, Rochester, MN
2013-present Professor of Pathology, Mayo Clinic, Rochester, MN

C. Contribution to Science

Lynch Syndrome: I collaborated with Henry Lynch on multiple descriptive studies covering clinical and pathologic features of Lynch syndrome, written before the genetics of that condition were known. One review has been cited more than 1000 times. (1) Jeremy Jass and I collaborated on a histology paper that got the pathology of Lynch Syndrome largely correct. (2) I attended the Bethesda conference in 1996 and collaborated on the publication of the Bethesda criteria. (3) I designed, performed and wrote the study characterizing tumor infiltrating lymphocytes as a robust predictor of microsatellite instability status. (4)

1) Lynch HT, Smyrk TC, Watson P et al. Genetics, natural history, tumor spectrum and pathology of hereditary nonpolyposis colorectal cancer: an updated review. Gastroenterology 1993;104:1535-1549. PMID: 8482467
2) Jass JR, Smyrk TC, Stewart SM, Lane MR, Lanspa SJ, Lynch HT. Pathology of hereditary non-polyposis colorectal cancer. Anticancer Res 1994;14:1631-1634. PMID: 7979198
3) Rodriguez-Bigas MA, Boland CR, Hamilton SR et al. A National Cancer Institute workshop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. J Natl Cancer Inst 1997;89:1758-1762. PMID: 9392616
4) Smyrk TC, Watson P, Kaul K, Lynch HT. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer 2001;91:2417-2422. PMID: 11413533

Eosinophilic esophagitis: We published the first description of eosinophilic esophagitis as a syndrome involving dysphagia. (1) My interest in that condition has continued over the years, resulting in contributions addressing whether diagnostic criteria are too strict (2), evaluating new diagnostic techniques (3) and elucidating the pathophysiology. (4)

1) Attwood SE, Smyrk TC, DeMeester TR, Jones JB. Esophageal eosinophilia with dysphagia. A distinct clinicopathologic syndrome. Dig Dis Sci 1993;38:109-116. PMID: 8420741
2) Ravi K, Talley NJ, Smyrk TC et al. Low grade esophageal eosinophilia in adults: an unrecognized part of the spectrum of eosinophilic esophagitis? Dis Dis Sci 2011;56:1981-1986. PMID: 21298480
3) Katzka DA, Geno DM, Ravi K et al. Accuracy, safety and tolerability of tissue collection by Cytosponge vs endoscopy for evaluation of eosinophilic esophagitis. Clin Gastroenterol Hepatol 2015;13:77-83. PMID: 24997328
4) Katzka DA, Ravi K, Geno DM et al. Endoscopic mucosal impedance measurements correlate with eosinophilia and dilation of intercellular spaces in patients with eosinophilic esophagitis. Clin Gastroenterol Hepatol 2015. PMID: 25592662

IgG4-related disease: Our 2003 paper was the first to clearly separate two forms of autoimmune pancreatitis, one IgG4-related and one not. (1) That separation is now widely accepted. We also had an early paper described the role of tissue IgG4 in the diagnosis. (2) I participated in the first international consensus conference on IgG4-related disease and contributed to the publication of its findings. (3, 4)

1) Notohara K, Burgart LJ, Yadav D, Chari S, Smyrk TC. Idiopathic chronic pancreatitis with periductal lymphoplasmacytic infiltration: clinicopathologic features of 35 cases. Am J Surg Pathol 2003;8:1119-1127. PMID: 12883244
2) Zhang L, Notohara K, Levy MJ, Chari ST, Smyrk TC. IgG4-positive plasma cell infiltration in the diagnosis of autoimmune pancreatitis. Mod Pathol 2007;20:23-28. PMID: 16980948
3) Stone JH, Khosroshahi A, Despande V et al. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. Arthritis Rheum 2012;64:3061-3067. PMID: 22736240
4) Deshpande V, Zen Y, Chan JK et al. Consensus statement on the pathology of IgG4-related disease. Mod Pathol 2012;25:1181-1192. PMID: 22596100

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/thomas.smyrk.1/bibliography/47997356/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

8702.02-A1 P50CA 102701 Petersen (PD/PI) 9/18/14-8/31/19
NCI
Mayo Clinic SPORE in Pancreatic Cancer – Core 2: Tissue Core
Build and administrate the pancreatic tissue core.
Role: Co-Investigator

FP00051409 P30DK 84567 LaRusso (OP) 9/1/14-8/31/19
NIDDK
Mayo Center for Cell Signaling in Gastroenterology
This center core grant correlates research requests with appropriate tissue sources.
Role: Other Professional

FP00062460 CA 163059 Wang (CI) 9/21/11-8/31/16
NIH
Validation Core C ? U MI Betmet Core
This study assesses new imaging techniques for dysplastic Barrett's esophagus.
Role: Co-Investigator

18248.02 U01DK 74008 Farrugia (CI) 9/1/12-8/31/16
NIDDK
Gastroparesis Clinical Research Consortium – Data Coordinating
This major goal is to study the pathology gastroparesis.
Role: Co-Investigator

FP00064454 Limburg (CI) 9/24/12-9/23/19
HHSN2612012000421
NIH
Cancer Prevention Agent Development Program: Early Phase Clinical Research
The major goal is to use pathology to assess response to chemoprevention.
Role: Co-Investigator

FP00067546 U01AA 21788 Shah (CI) 9/20/12-6/30/17
NIH
TREAT – Mayo
This major goal is to determine prognostic markers for alcoholic liver disease.
Role: Co-Investigator

FP00062052; CA 163004 Wang (CI) 9/26/11-8/31/16
NIH
Stem Cells and the Origins of Barrett's Esophagus
This major goal is to identify esophageal stem cells and delineate their role in Barrett's esophagus.
Role: Co-Investigator

Completed Research Support

n/a

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Urrutia, Raul

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor of Medicine, Biophysics, and Biochemistry & Molecular Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Cordoba, Cordoba, Argentina	Fellowship	05/1984	Biochemistry
University of Cordoba, Cordoba, Argentina	M.D.	05/1987	Tumor Biology

A. Personal Statement:

I received my basic science training at the University of Cordoba, Argentina where I performed my first studies on pancreatic cancer. Subsequently, I came to the United States to work in the area of cell motility and migration at the National Institute of Health (NIH), Bethesda, MD. Subsequently, I joined the faculty of the Mayo Clinic where I still continue my research in pancreatic cancer. Thus, my scientific dedication to cancer has remained a continuum for almost 30 years. Our laboratory has pioneered basic science studies in the area of transcription, chromatin, and epigenetics in Gastrointestinal cancer and other human diseases, including metabolic, neuropsychiatric, immunological, vascular, and auditory. Relevant to the current application, our work on KLF transcription factors extended into the characterization of many chromatin co-factors, which function as writer, reader, and erasers of the histone code. In particular, I have an active line of investigation on EZH2, because of which I have become collaborator of the current proposal. I have mentored more than 30 graduate and postgraduate fellows who have gone on to have independent laboratories. Over the years at Mayo, I have served as Director of the GI Research Unit, Director of the Ph.D. Program in Tumor Biology, Associate Director for Genomics at the Mayo Clinic General Clinical Research Center (GCRC), and Director of the GI Cancer Research Program at the Mayo Cancer Center and member of the NIDDK GI Center, the Mayo Pancreatic Cancer Spore, and the Mayo Epigenetic Interest group. Currently, I am part of the Translational Epigenomic Program in the Center for Individualized Medicine, for which I serve as Director for Education and Academic Relationships. Our mission is to promulgate the study and application of epigenetics and epigenomics to medicine, mechanistically and translationally. Regarding interactions with the extramural GI and pancreatic community, I have been council of the American Pancreatic Association, serving also as its President in 2008. I have also been on the council of the International Association of Pancreatology (IAP) and American Gastroenterology Association (AGA). Furthermore, I have served as the Vice-Chair and later chair of the pancreatic section of American Gastroenterological Association. I have served as the Editor-in-Chief for three journals in the field, including *Pancreatology*, *International Journal of Gastrointestinal Cancer*, and *Case Reports in Gastroenterology.* In summary, I have an established record of successful and productive research projects regarding transcriptional regulation, epigenetics, and chromatin dynamics. My laboratory is located in the highly collaborative environment of the GI Research Unit at Mayo in adjacency to the PI of the current grant, with whom I have the pleasure to collaborate intensively. Thus, I will be helpful for planning and interpreting experiments included in the current grant.

1. Grzenda A, Lomberk G, Svingen P, Mathison A, Calvo E, Iovanna J, Xiong Y, Faubion W, **Urrutia R**. Functional Characterization of EZH2beta reveals the increased complexity of EZH2 isoforms involved in the regulation of mammalian gene expression. *Epigenetics Chromatin.* 2013; 6(1):3. PMCID: PMC3606351

2. Xiong Y, Khanna S, Grzenda AL, Sarmento OF, Svingen PA, Lomberk GA, **Urrutia RA**, Faubion WA Jr. Polycomb Antagonizes p300/CREB-binding Protein-associated Factor to Silence FOXP3 in a Kruppel-like Factor-dependent Manner. *J Biol Chem*. 2012 Oct 5;287(41):34372-85.

3. Mitic T, Caporali A, Floris I, Meloni M, Marchetti M, **Urrutia R**, Angelini GD, and Emanueli C. EZH2 modulates angiogenesis in vitro and in a mouse model of limb ischemia. Mol Ther. 2015 Jan;23(1):32-42

4. Floris I, Descamps B, Vardeu A, Miticc T, Posadino AM, Shantikumar S, Sala-Newby G, Capobianco G, Mangialardy G, Howard L, Dessole S, **Urrutia R**, Pintus G, Emanueli C. Gestational diabetes mellitus impairs fetal endothelial cell functions through a mechanism involving microRNA-101 and histone methyltransferase enhancer of zester homolog-2. Arterioscler Thromb Vasc Biol. 2015 Mar;35(3):664-74.

B. Positions and Honors

Positions and Employment

1987-1989	Visiting Fellow, Laboratory of Molecular Otology, NIDCD, National Institutes of Health, Bethesda, MD
1989-1990	Visiting Fellow, Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD
1990-1991	Visiting Associate at the Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD
1991-1992	Visiting Associate, Laboratory of Cellular Biology, NIDCD National Institutes of Health, Bethesda, MD
1992-1994	Research Associate, Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN
1994-2000	Assistant Professor of Medicine, College of Medicine, Mayo Clinic, Rochester, MN
1995-	Assistant Professor, Tumor Biology, Mayo Graduate School, Mayo, Clinic, Rochester, MN
1996-	Assistant Professor of Biochemistry and Molecular Biology, College of Medicine, Mayo Clinic, Rochester, MN
1997-2004	Council, International Association of Pancreatology
1998-2002	Senior Associate Consultant, Mayo Clinic Cancer Center and GI Research Unit, Mayo Clinic, Rochester, MN
1998-2007	Council, American Pancreatic Association
2000-2011	Chair, GI Research Unit, Mayo Clinic, Rochester, MN
2000-2004	Education Coordinator, Tumor Biology Program, Mayo Graduate School, Mayo Clinic, Rochester, MN
2000-	Professor of Medicine, College of Medicine, Mayo Clinic, Rochester, MN
2001-2002	Genomics Program Director, General Clinical Research Center, Mayo Clinic, Rochester, MN
2002-	Consultant, Mayo Clinic Cancer Center and GI Research Unit, Mayo Clinic, Rochester, MN
2002-2005	Director, GI Cancer Group, Mayo Clinic Cancer Center, Mayo Clinic, Rochester, MN
2003-2004	Vice-chair, Pancreatic Diseases Section, American Gastroenterological Association
2005-2010	Editor-in-Chief, *Pancreatology*
2005-	Professor of Biophysics, College of Medicine, Mayo Clinic, Rochester, MN
2004-2006	Chair, Pancreatic Diseases Section, American Gastroenterological Association
2006-2007	President, American Pancreatic Association
2006-2009	Editor-in-Chief, *Journal of Gastrointestinal Cancer*
2007-	Editor-in-Chief, *Case Reports in Gastroenterology*
2008-2011	Director, ACS/IRG, Mayo Clinic Cancer Center, Mayo Clinic, Rochester, MN
2012-	Director for Education and Academic Relationships, Translational Epigenomic Program, Center for Individualized Medicine, Mayo Clinic, Rochester, MN

Honors and Awards

1987	Magna Cum Laude, Medical Doctor
1995	Cancer Award from Fraternal Order of Eagles

1996-1997	Cancer Award from Fraternal Order of Eagles
1994-1999	NIH First Award, R-29, National Institutes of Health
1998-2002	NIDDK R01 Award
1999	Charlotte Geyer Foundation Award
2000	Charlotte Geyer Foundation Award
2000	NIH R01, National Institutes of Health
2002	The Lustgarten Foundation for Pancreatic Cancer Research
2002-2008	NIDDK R01 Renewal, National Institutes of Health
2009-2014	NIDDK R01 Renewal, National Institutes of Health
2014-2019	NIDDK R01 Renewal, National Institutes of Health

C. Contribution to Science

1. I graduated as an M.D. Magna Cum Laude in 1987 from the University of Cordoba Medical School in Cordoba, Argentina, where I initiated my studies in the area of pancreatic cancer. To pursue training in this field, I joined the National Institutes of Health from where I published work that lead to seminal discoveries in the area of mechanochemical translocators. My basic science studies on the molecular properties of these proteins demonstrated for the first time that kinesin transports organelles along microtubules. Subsequently, I co-discovered dynamin II and demonstrated that this protein, which was thought to be only a neuronal microtubule motor, was indeed an endocytic GTPase universally conserved form yeast to vertebrates. I also discovered myosin I E, which today is dubbed as “super myosin” due to its ability to support rapid actin-based movement. Together, these proteins are the best known ATP and GTP hydrolyzing enzymes that support cancer-associated processes by critically regulating cell motility, migration, organelle transport, chromosome movements, and signaling.

    a. **Urrutia R**, McNiven MA, Albanesi JP, Murphy DB, Kachar B. Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. *Proc Natl Acad Sci USA.* 1991; 88:6701-6705. PMCID: PMC52156
    b. Torre E, McNiven MA, **Urrutia R**. Dynamin 1 antisense oligonucleotide treatment prevents neurite formation in cultured hippocampal neurons. *J Biol Chem*. 1994; 269(51):32411-7. PMID: 7798241
    c. Cook TA, **Urrutia R**, McNiven MA. Identification of Dynamin 2, an isoform ubiquitously expressed in rat tissues. *Proc Natl Acad Sci USA.* 1994; 91:644-648. PMCID: PMC43005
    d. **Urrutia R**, Jung G, Hammer JA III. The Dictyostelium myosin IE heavy chain gene encodes a truncated isoform that lacks sequences corresponding to the actin binding site in the tail. *Biochim Biophys Acta.* 1993; 1173:225-229. PMID: 8504170

2. With the initial goal of defining how these proteins regulate cancer processes, I began my studies on transcriptional regulation, which led us to change gears in our research and pioneer the field of KLF proteins. While KLFs have gained significant attention as transcription factor proteins that play a significant role in nuclear reprogramming (KLF4 is one of the best known Yamanaka factors for iPS cells), these proteins play a pleotropic role in the pathobiology of many diseases. My laboratory discovered a family of KLF proteins that work at the intersection of metabolism and cancer. Specifically, we cloned KLF10, KLF11, KLF13, KLF14, and KLF16, which function as metabolic regulators and tumor suppressor proteins. We demonstrated that while germ line mutation of these proteins cause metabolic diseases, such as diabetes, their epigenetic silencing contributes to the process of pancreatic carcinogenesis.

    a. Fernandez-Zapico ME, Mladek A, Ellenrieder V, Folch-Puy E, Miller L, **Urrutia R**. An mSin3A interaction domain links the transcriptional activity of KLF11 with its role in growth regulation. *EMBO J.* 2003; 22(18):4748-58. PMCID: PMC212736
    b. Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, Vaillant E, Benmezroua Y, Durand E, Bakaher N, Delannoy V, Vaxillaire M, Cook T, Dallinga-Thie GM, Jansen H, Charles MA, Clement K, Galan P, Hercberg S, Helbecque N, Charpentier G, Prentki M, Hansen T, Pedersen O, **Urrutia R**, Froguel P. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. *Proc Natl Acad Sci USA.* 2005; 102(13):4807-12. PMCID: PMC554843

c. Bonnefond A, Lomberk G, Buttar N, Busiah K, Vaillant E, Lobbens S, Yengo L, Dechaume A, Mignot B, Simon A, Scharfmann R, Neve B, Tanyolac S, Hodoglugil U, Pattou F, Cave H, Iovanna J, Stein R, Polak M, Vaxillaire M, Froguel P, **Urrutia R**. Disruption of a novel KLF-p300-regulated pathway for insulin biosynthesis revealed by studies of the c.-331 INS mutation found in neonatal diabetes mellitus. *J Biol Chem.* 2011; 286(32):28414-24. PMCID: PMC3151084
d. Lomberk G, Grzenda A, Mathison A, Escande C, Zhang JS, Calvo E, Miller LJ, Iovanna J, Chini EN, Fernandez-Zapico ME, **Urrutia R**. Krüppel-like factor 11 regulates the expression of metabolic genes via an evolutionarily conserved protein interaction domain functionally disrupted in maturity onset diabetes of the young. *J Biol Chem*. 2013; 288(24): 17745-58. PMCID: PMC3682574

3. Attempts to better understand how KLF proteins work, my research began to characterize on how they couple to chromatin and mediate epigenetic signaling. We focused on testing the histone code hypothesis postulated by David Allis, which proposed that the type and combination of posttranslational modifications, known as histone marks, were sufficient to function as a predictive epigenetic code. As postulated, this hypothesis did not take into consideration the impact that the regulation of writers, readers, and erasers of these marks by themselves had on epigenetic inheritance. However, my studies demonstrated that posttranslational modifications in the readers of these marks acted as additional “subcodes”, which were necessary for gene silencing. By working on histone code erasers, we discovered Sin3-Interacting Domains, which are protein motifs that function as a link between DNA-bound transcription factors and histone deacetylases (HDACs). Subsequent work established that these pathways are antagonized by writer enzymes such as HATs and HMTs, including the identification of novel EZH2 proteins, which are among the best epigenetic oncogenes known to date.

a. Lomberk G, Bensi DE, Fernandez-Zapico ME, **Urrutia R**. Evidence for the existence of an HP1-mediated subcode within the histone code. *Nat Cell Biol.* 2006; 8: 407-15. PMID: 16531993
b. Zhang J-S, Moncrieffe MC, Kaczynski J, Ellenrieder V, Prendergast FG, **Urrutia R**. A conserved α-helical motif mediates the interaction of Sp1-like transcriptional repressors with the corepressor mSin3A. *Mol Cell Biol.* 2001; 21:5041-5049. PMCID: PMC87230
c. Grzenda A, Lomberk G, Svingen P, Mathison A, Calvo E, Iovanna J, Xiong Y, Faubion W, **Urrutia R**. Functional Characterization of EZH2beta reveals the increased complexity of EZH2 isoforms involved in the regulation of mammalian gene expression. *Epigenetics Chromatin.* 2013; 6(1):3. PMCID: PMC3606351
d. Lomberk G, Mathison AJ, Grzenda A, Seo S, DeMars CJ, Rizvi S, Bonilla-Velez J, Calvo E, Fernandez-Zapico ME, Iovanna J, Buttar NS, **Urrutia R**. Sequence-specific recruitment of heterochromatin protein 1 via interaction with Krüppel-like factor 11, a human transcription factor involved in tumor suppression and metabolic diseases. *J Biol Chem*. 2012; 287(16):13026-39. PMCID: PMC3339955

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/raul.urrutia.1/bibliography/40845204/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

R01 DK052913 (Urrutia, PI) 09/01/2014-08/31/2019
NIDDK
The Role of Zinc Finger Co-Factors in Pancreatic Cell Growth
The major goal of this project is to determine the role of novel zinc finger transcription factors, KLFs, and their co-factors in pancreatic cell growth.
Role: Principal Investigator

[redacted]

[redacted]

P50 CA102701 (Petersen, PI) 09/01/2014-08/31/2015
NIH-NCI – Mayo Clinic Pancreatic Cancer SPORE
Pilot Project Award: EZH2-Based Experimental Therapeutics in Pancreatic Cancer
The major goal of this project is to characterize the effect of EZH2 downstream of oncogenic pathways on growth-promoting gene networks to facilitate PDAC initiation and promotion in a manner that is amenable to pharmacological inhibition.
Role: Principal Investigator of Pilot Project

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
First Name*: William
Middle Name: A[redacted]
Last Name*: Faubion
Suffix: MD

2. Human Subjects

Clinical Trial? ● No ❍ Yes
Agency-Defined Phase III Clinical Trial?* ● No ❍ Yes

3. Permission Statement*

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

❍ Yes ● No

4. Program Income*

Is program income anticipated during the periods for which the grant support is requested? ❍ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

Budget Period*	Anticipated Amount ($)*	Source(s)*

Tracking Number: GRANT11954038
Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Cover Page Supplement

5. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ● No ❍ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

6. Inventions and Patents (For renewal applications only)

Inventions and Patents*: ❍ Yes ● No

If the answer is "Yes" then please answer the following:

Previously Reported*: ❍ Yes ❍ No

7. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

First Name*:

Middle Name:

Last Name*:

Suffix:

❑ Change of Grantee Institution

Name of former institution*:

PHS 398 Modular Budget

OMB Number: 0925-0001

Budget Period: 1

Start Date: 07/01/2016 End Date: 06/30/2017

A. Direct Costs

	Funds Requested($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Arif Karim and Narendra Gan[redacted]

Indirect Cost Rate Agreement Date: 01/20/2015

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A+B)

Funds Requested ($): [redacted]

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Modular Budget

Budget Period: 2

Start Date: 07/01/2017 End Date: 06/30/2018

A. Direct Costs

	Funds Requested($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Arif Karim and Narendra Gan[redacted]

Indirect Cost Rate Agreement Date: 01/20/2015

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

Tracking Number: GRANT11954038

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Modular Budget

Budget Period: 3

Start Date: 07/01/2018 End Date: 06/30/2019

A. Direct Costs

	Funds Requested($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Arif Karim and Narendra Gan[redacted]

Indirect Cost Rate Agreement Date: 01/20/2015

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

Tracking Number: GRANT11954038

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Modular Budget

Budget Period: 4

Start Date: 07/01/2019 End Date: 06/30/2020

A. Direct Costs

	Funds Requested($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Arif Karim and Narendra Gan[redacted]

Indirect Cost Rate Agreement Date: 01/20/2015

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

Tracking Number: GRANT11954038

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Modular Budget

Budget Period: 5

Start Date: 07/01/2020 End Date: 06/30/2021

A. Direct Costs

	Funds Requested($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Arif Karim and Narendra Gan[redacted]

Indirect Cost Rate Agreement Date: 01/20/2015

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A+B)

Funds Requested ($): [redacted]

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium F&A for Entire Project Period ($)	[redacted]
Section A, Total Consortium F&A for Entire Project Period ($)	0.00
Section A, Total Direct Costs for Entire Project Period ($)	[redacted]
Section B, Total Indirect Costs for Entire Project Period ($)	[redacted]
Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period ($)	[redacted]

2. Budget Justifications

Personnel Justification	Personnel_Justification_1_.pdf
Consortium Justification	
Additional Narrative Justification	

Tracking Number: GRANT11954038

Funding Opportunity Number: PA-13-302. Received Date: 2015-07-02T14:04:51.000-04:00

PERSONNEL JUSTIFICATION:

Senior/Key Personnel:
Faubion, WA Jr. MD, PI, (30%) 3.6 person months/year over the life of the grant. As Principal Investigator, Dr. Faubion is responsible for the daily conduct of the proposed studies. Dr. Faubion received his medical degree from the University of Texas Health Science Center, Houston, Tx, and his GI subspecialty training at Mayo Clinic, Rochester, MN. He received basic immunology training in the laboratory of Dr. Cox Terhorst, Beth Israel, Harvard, MS in 2000-2003. Dr. Faubion is an expert in cellular immunology, murine models of colitis, and specifically T regulatory cell biology, and FOXP3 gene regulation. As a member of the Epigenetic and Chromatin Dynamics Laboratory, he is intensely focused on the epigenetic regulation of FOXP3 dependent gene networks. Dr. Faubion has the expertise and his laboratory has the appropriate methodology to support the feasibility of the current proposal.

Collaborators:
Urrutia, Raul A. MD, Collaborator, no measurable effort. Dr. Urrutia, as the director of the Chromatin and Epigenetics Laboratory at Mayo Clinic, already provides intellectual input. He has provided and will continue to provide a variety of unique and critical reagents such as the EZH2 SET domain mutant and EZH2 HMT pharmacologic inhibitors. His letter of support confirms his willingness to collaborate.
Smyrk, Thomas C. MD, Collaborator, no measurable effort. Dr. Smyrk has agreed to provide pathologic expertise in reviewing the histologic sections of mouse colon and to provide a histologic colitis score. His letter of support confirms his willingness to collaborate.
Pasare, Chandrashekhar, PhD, UTSW Collaborator, no measurable effort. Dr. Pasare is an Associate Professor in the Department of Immunology, UTSW. He has extensive experience in IL6 signaling pathways in relationship to Treg function. He has already provided intellectual input and the IL6R signaling mutant mouse line. His letter of support confirms his willingness to collaborate.
Ekker, Stephen PhD, Collaborator, no measurable effort. Dr. Ekker is a Professor of BMB and an expert in gene editing technology. He has already provided intellectual input and TALEN constructs. His willingness to collaborate on Aim 3 is indicated in his letter of support.

Other Personnel:
Svingen, P, Technician, 12 person months/year for years 1-5. Mrs. Svingen is a senior laboratory technician with over 30 years experience at Mayo Clinic. She has accomplished in areas of cellular immunology including T cell subset isolation, culture, cell transfection techniques, and flow cytometry. She also is capable of genomic DNA isolation and ChIP assay. Furthermore, she is proficient with animal colitis models such as bone marrow transfer, adoptive T cell transfer, DSS colitis, and TNBS colitis.
Xiong, Y, Technician, 3.78 person months/year for years 1-5. Dr. Xiong is an MD/PhD with experience in molecular biology, ChIP assay, and DNA and RNA isolation from colonic T cell subsets.
His efforts will be focused on the maintenance of the genome integrated FLP cell lines and the generation of new constructs and recombinant fusion proteins.
Lastly, our laboratory is highly interactive and these individuals will collaborate on each of the specific aims. Their data is presented once a week at lab meetings and periodically at retreats as well as national and international meetings. Therefore, the high coherence of this group and their exquisite training make them among the most qualified researchers possible to participate in this grant.

PHS 398 Research Plan

Please attach applicable sections of the research plan, below.

OMB Number: 0925-0001

1. Introduction to Application (for RESUBMISSION or REVISION only)	Introduction_Final.pdf
2. Specific Aims	HYPOTHESIS_AND_SPECIFIC_AIMS.pdf
3. Research Strategy*	Research_Plan.pdf
4. Progress Report Publication List	Publication_List3.pdf
Human Subjects Sections	
5. Protection of Human Subjects	
6. Inclusion of Women and Minorities	
7. Inclusion of Children	
Other Research Plan Sections	
8. Vertebrate Animals	Vertebrate_Animals.pdf
9. Select Agent Research	
10. Multiple PD/PI Leadership Plan	
11. Consortium/Contractual Arrangements	
12. Letters of Support	letters_combined.pdf
13. Resource Sharing Plan(s)	Resource_Sharing_Plan.pdf
Appendix (if applicable)	
14. Appendix	

INTRODUCTION TO APPLICATION

We appreciate the highly laudatory review on 2/19/2015 of our proposal (21st percentile). The constructive critique has resulted in the addition of **extensive new preliminary data** and the development of **new reagents and animal models**, which have improved the proposal in novelty, relevance, feasibility, design, and methodology. Below, we address both convergent suggestions as well as individual points not shared by all reviewers (revisions highlighted in yellow in newproposal).

Productivity: Since the last review, 3 additional senior authored manuscripts have been published totaling **10** peer reviewed publications generated through the experiments defined in the first four years of this award. Regarding the concern that "this new proposal is a major departure from the directions proposed in the previous proposal," we have provided in detail (progress report) the continuum of logic flowing from the previous aims (KLF binding to DNA, coupling to epigenetic machinery, and with relevance to colitis) to the current hypothesis. In fact, this grant is focused on the epigenetic machinery (EZH2) identified in previous Aim3 and the relevance to Treg biology and colitis.

Key preliminary data: There was consensus among reviewers on the need for additional data.
(i) It was noted that, "Although the colitis T cell transfer model is primarily used in the proposal, the preliminary results use the DSS model." We have developed a new tamoxifen-inducible CRE-EZH2 KO animal and demonstrated a requirement for EZH2 in FOXP3+ cells for prevention of Rb^{hi} mediated colitis, a critical control for the *in vivo* mutational analysis (Aim3).
(ii) We were queried whether the "FOXP3dEZH2 mice show spontaneous colitis?" We have yet to age mice beyond 8 weeks; yet we show the susceptibility to subclinical (1%DSS) colitis in newexperiments.
(iii) More data in support of A2 was required, as "The model that EZH2 phosphorylation … is critical in disrupting Treg cell function is highly speculative." We have developed an experimental pipeline that consists of mass spectrophotometry, linear motif analyses, molecular modeling, mutational screens, and functional assays to definitively test the hypothesis of Aim 2. First, mass spectrophotometry identified phosphorylated residues predicted by molecular modeling to regulate EZH2 HMT activity. Extensive linear motif analysis predicted 6 such residues to be regulated by MAPK and Jak kinases. We generated 12 mutant EZH2 constructs and a CRISPR/Cas9 EZH2 mutant cell line in which to test the effect of phosphorylated residues on EZH2 HMT activity. Exemplary of this pipeline, we discovered phosphorylation of Y641 by Jak2 to regulate EZH2 activity. As we were queried in several ways for the effect of IL10 in this mechanism, we have incorporated IL10 into the tightly focused dissection of the critical inflammatory pathways leading to reversible modifications of EZH2 HMT activity.

Individual points not shared by all reviewers and not addressed above:
Reviewer 1 (R1) noted previous data exists regarding IL6 disruption of Treg function. Indeed, IL6 dependent *T effector cell* ***resistance*** to Treg suppression is well reported (Pasare and Medzhitov 2003, Viglietta, Baecher-Allan et al. 2004, Haas, Hug et al. 2005, Venken, Hellings et al. 2006); however purported mechanisms involve intrinsic T effector cell pathways, not Treg cell biology (Schenten, Nish et al. 2014, Bhela, Kempsell et al. 2015). The conversion of FOXP3+ cells to TH17 cells downstream of IL6 signaling has been reported (Komatsu, Okamoto et al. 2014); yet again, **the mechanism(s) leading to Treg cellular conversion is unknown, represents a critical gap in knowledge, and addressed in this proposal**. **R1** also requested a broader description of outcome variables to assess *in vivo* studies and a discussion regarding the role for EZH2 in Stat3 activity. Both are provided in the revised proposal. **Reviewer 2** (R2) queried "whether EZH2 may repress cytokines in other lymphocytes," and that we "examine cell surface molecules and cytokines, such as IL-10, … to determine whether they are also affected by EZH2 blockade." We demonstrate flow analysis of surface molecules and the results of multiplex ELISA for cytokine production. Furthermore, we have explored a role for EZH2 in T effector cells in alternative approaches. **R2** noted A3 to be "wide ranging with superficial discussion of pitfalls and alternative approaches." We have streamlined the aim by exclusion of the neonatal transfer experiment (feasibility was questioned) and have greatly enhanced the Pitfalls and Alternative Approaches. ***Summary***: In response to this excellent critique, we have provided evidence of continued productivity, preliminary data from three new major lines of experimentation, developed two new mouse models, and addressed each review point by point with modifications to the proposal.

HYPOTHESIS AND SPECIFIC AIMS:

The transcription factor FOXP3 is critical to the regulation of numerous debilitating human immune-mediated diseases, the prevalence of which together affect over 8.5 million people (1 in 31 U.S. residents). In Inflammatory Bowel Disease (IBD) chronic intestinal inflammation indicates aberrant *in vivo* FOXP3+ T regulatory (Treg) cell function (1). Similarly, proinflammatory signals *in vitro* impair Treg function (2). Our lab was the first to characterize the essential role for the histone methyltransferase (HMT) EZH2 in the epigenetic regulation of FOXP3 (3). Recent published work extended our observations indicating a key role for EZH2 in FOXP3 repressor function (4); however the regulation and biological impact of the FOXP3-EZH2 pathway to IBD is unknown. This knowledge is important given the apparent loss of function of Treg cells in inflammation.

Our *long-term goal* is to dissect epigenetic mechanisms regulating Treg cellular differentiation and function, particularly within the setting of GI inflammatory diseases; as these discoveries will facilitate design of human cell therapy trials for IBD. Consequently, the *objective* of this grant is to characterize the role for the epigenetic regulator EZH2 in Treg suppressive function. These investigations are strongly supported by preliminary data demonstrating that: 1) EZH2 is required for Treg suppressive function; 2) IL6 signaling leads to phosphorylation and inhibition of EZH2; 3) lymphocytes isolated from the intestine of IBD patients demonstrate activation of IL6-induced gene networks and loss of EZH2 HMT function; and 4) conditional knockout of EZH2 in FOXP3+ T cells leads to *in vivo* immune dysfunction. Based upon these compelling data we propose the **CENTRAL HYPOTHESIS that EZH2 plays a critical role in the homeostasis of Treg cells, and the disruption of EZH2 function by inflammatory signaling pathways contributes to IBD.** Our rationale is that identification of the mechanism(s) to restore Treg suppressive function in the setting of intestinal inflammation will offer new therapeutic opportunities within the field of IBD. Our specific aims will test the following hypotheses:

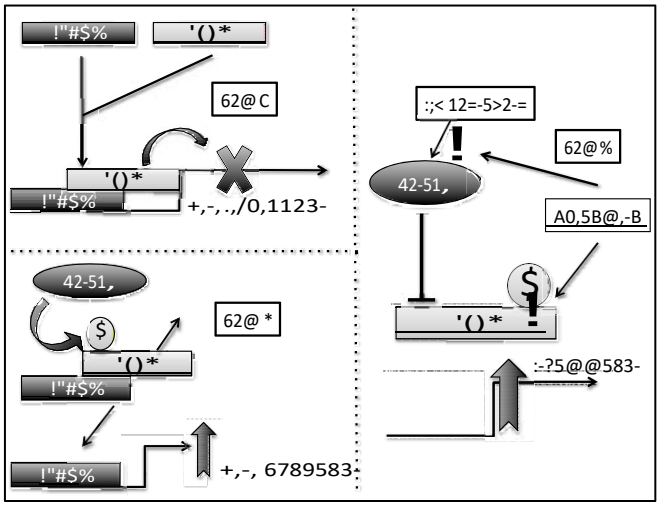


Figure 1: Conceptual framework. Through the mechanistic experiments designed in the following aims we will identify the role for FOXP3 in the recruitment of EZH2 to core target genes required for Treg function (Aim 1). We will define the signaling network responsible for phosphorylation of EZH2 and disrupted HMT function (Aim 2). Finally, we will perform pre-clinical studies of innovative therapy designed to generate Treg cells resistant to disruptive modifications in the setting of inflammation (Aim 3).

Aim 1: Repression of immunoregulatory gene networks by FOXP3 requires the formation of a complex between this transcription factor and EZH2.

Aim 2: Inflammatory stimuli, such as IL6 lead to EZH2 phosphorylation and thereby disrupt the enzymatic activity of this epigenomic regulator.

Aim 3: Inhibition of the IL6 to EZH2 signaling pathway permits sustained Treg suppressive function in the setting of intestinal inflammation.

Upon conclusion, we will understand the role for EZH2 in Treg loss of function in the setting of active inflammation. This discovery will stimulate new areas for experimental therapeutics in human chronic inflammatory diseases. Our environment in the Epigenetic and Chromatin Dynamics Laboratory combined with the Department of Immunology at the Mayo Clinic makes us uniquely qualified to pursue this objective given the extensive collective experience of histone methyltransferase biology, proinflammatory signaling networks, and *FOXP3* gene regulation.

RESEARCH STRATEGY

***Significance**:* FOXP3 is a transcription factor critical to the development, maintenance, and function of Treg cells. FOXP3 repression of key target gene networks is required for maintenance of Treg function (4,5). Key observations performed during the last funding cycle of this grant led us to identify for the first time a key role for EZH2 in the regulation of FOXP3 expression (3). The significance of this discovery is evident in the fact that subsequently three groups have confirmed and extended our observations on the import of EZH2 in the differentiation and maintenance of Treg cells (4,6,7). Yet, actionable mechanistic insight into 1) the FOXP3 EZH2 protein complex, 2) inflammatory signaling regulating EZH2 activity, and 3) the potential for EZH2-based therapy in IBD represents a critical gap in knowledge addressed in this application.

The canonical function of EZH2 is trimethylation of lysine 27 (K27) of histone 3 (H3) (H3K27me3). This "histone mark" leads to chromatin inaccessibility and gene silencing (8). EZH2 undergoes post-translational modifications downstream of metabolic, cell proliferative, and inflammatory signaling pathways (9-13). We have now discovered that proinflammatory pathways lead to EZH2 phosphorylation and inhibit EZH2 histone methyltransferase (HMT) activity in primary lymphocytes. This discovery is significant, as inflammatory signals can disrupt Treg function (2,14-16), which is apparent within the inflamed environment of IBD and characterized by the co-expression of the proinflammatory cytokines IFNγ and IL17 (17,18). However, little still is known about the mechanism(s) of inflammation-induced disruption of Treg function. Thus, the current study will characterize the role for inflammation-induced modification of EZH2, the inhibition of HMT activity, and the subsequent disruption of Treg suppressive function. *This contribution is significant since it will establish that several pathways targeted by available therapies (i.e. IL1β, IL6, TNFα) have the potential to regulate EZH2 HMT activity through post-translational modifications. Furthermore, current Treg cell therapy trials (19), while promising have not addressed the key issue of in vivo inflammation-induced disruption of Treg function. This proposal seeks to address this knowledge gap.*

IBD, increasing in prevalence, represents a major national cost measured by both patient suffering and economic burden (as evidenced by over 600 original studies on cost of IBD). Despite significant advances in care (i.e. biologic therapeutics), clinical trial data demonstrate remission rates at best of 40%; thus there remains an enormous unmet medical need. The clinical significance of our study will be the opening of a new avenue in therapeutics directed at stimulation of autologous Treg cells to function within the inflammatory milieu. Our results will impact not only IBD, but also human immune mediated disease broadly.

***Innovation**:* Previous work on epigenetic mechanisms in Treg function has largely focused on genome wide associations using ChIP-Seq methodology (4). *The proposed research is innovative because we investigate the effect <u>of inflammatory signaling pathways</u> on epigenetic complexes in Treg cells, a heretofore-unexamined process. Our experimental approach dives more deeply, biochemically and biologically into the mechanistic impact of this pathway beyond the simple catalog of gene targets provided by the previous ChIP-Seq methodology. Insight into epigenetic mechanisms is impactful as T cell progenitor cells inherit the parent transcriptional profile and unlike genetic change, they are modifiable by currently available therapy. In fact, anti-EZH2 drugs are currently being tested in clinical trials and knowing the role of this HMT in T cells will become imperative as they become widely available in the clinical setting.* Conceptual innovations include: 1) the novel role for proinflammatory signaling networks in the regulation of the HMT EZH2 pathway in lymphocytes and IBD; 2) the discovery of the required partnership between FOXP3 and EZH2 in Treg function; and 3) *ex vivo* genetic engineering of Treg cells to function in the setting of inflammation. Methodologically, there are four new animal models used in this application including the conditional EZH2 KO mouse line, two inducible EZH2 KO mouse lines, and the conditional IL6Rα knockout mouse line. The *in vivo* experimental techniques utilized in this proposal, particularly the cell transfer experiments utilizing adenoviral replacement of EZH2 and mutant constructs are state-of-the-art. Additional state-of-the-art methodology is provided by the use of CAS/CRISPR mutant cell lines and proteomic experiments, molecular mechanics and molecular dynamic techniques for studying the effects of post-translational modifications on the EZH2 protein, which combined are highly innovative in the field of immunology. In summary, the hypotheses guiding the proposal are novel and will be tested using a comprehensive approach with sophisticated methodology also giving rise to reagents, including new cell and animal models which will be widely shared with the scientific community.

Approach:

PROGRESS REPORT

General Statement: The beginning and ending dates of the current period of funding are 2/15/2011-1/31/2016. In this grant's previous cycle, 10 relevant original articles have been published.

Progress Towards Prior Specific Aims: Published Results. Our original hypothesis was that KLF family members, through coupling with chromatin modifying complexes regulate FOXP3 expression, and ultimately T reg cell function and colitis. Our most salient accomplishments during the prior funding period relevant to our prior three Specific Aims are provided below. The resulting publications are listed within the specified section of the application (see progress report publication list).

Prior Aim #1: KLF10 expression in T cells mediates a novel mechanism of resistance to colitis. As KLF10 is regulated by TGFβ and required for inducible FOXP3+ Treg cell (iTreg) development, we postulated that KLF10 is required in T cells to prevent immune mediated disease. KLF10 deficient animals are susceptible to colitis (#1 publication list) (3). Colitis susceptibility is transferred with the bone marrow indicating the requisite role for KLF10 in immune cells to prevent colitis (#2 publication list) (20). In lymphocytes, KLF10 regulates both FOXP3 and TGFβRII transcription generating a positive feedback loop in Treg development (3,21) (#3 publication list). This regulatory loop is relevant in murine models of colitis and encephalitis (20,21). Thus, KLF10 required for TGFβ induction of iTreg cells, functions in a positive feedback loop between TGFβ signaling and the TGFβRII promoter and is critical to immune regulatory function.

Prior Aim #2: KLF10 selectively regulates distinct genomic control regions of *Foxp3*. Because suspected KLF binding sites exist within the FOXP3 promoter locus, we postulated that distinct KLF binding domains regulate FOXP3 transcription. Using genome integrated cell lines, mutant mouse lines, and primary human cells, we identified novel KLF binding sites in both the core promoter and key enhancer domains. KLF10 binding to the core promoter recruits Sin3-HDAC or the HAT PCAF to repress or activate FOXP3 gene transcription, respectively (#1 and 2 publication list) (3,20). KLF14 binding to an enhancer domain represses FOXP3 through the heterochromatin protein 1 gene-silencing pathway (22)(#4 publication list), a relationship found to be generalizable to other model systems (#5 publication list) (23). FOXP3 domain functions discovered in the conduct of this aim led to the functional characterization of a private mutation causing familial IBD (#6 publication list) (24) and innovative methodology to quantify *in vivo* human Treg kinetics (#7 publication list) (25). Collectively, these observations identify distinct genomic control regions of FOXP3 through which KLF family members regulate FOXP3 transcription.

Prior Aim #3: KLF10 works via distinct chromatin remodeling required for *Foxp3* activation. Because KLF family members function to couple DNA binding to chromatin remodeling complexes, we examined the potential for KLF10 to regulate FOXP3 in an epigenetic fashion. We observed that KLF10 binding to the core promoter blocks recruitment of the **HMT, EZH2** thus preventing gene repression (#1 publication list) (3). We **discovered two structurally and functionally distinct EZH2 HMTs**; and both are capable of permanently silencing FOXP3 (#8 publication list) (26). Thus, KLF10 alternatively represses or activates FOXP3 through coupling with Sin3 or PCAF, and in the absence of KLF10, FOXP3 is permanently silenced by EZH2. In summary, we have successfully completed work on our previous specific aims demonstrating fidelity to the proposal. Additionally, our work into TGFβ signaling upstream of the FOXP3 promoter locus resulted in novel insights into hedgehog signaling pathways (#9 publication list) (27) and the function of an epithelial derived stem cell in colitis (28) (#10 publication list). In addition, by lending critical expertise gained from the funded studies, we were able to contribute to additional collaborative publications, further attesting to the success of our program during the past funding cycle (29-34).

This grant proposal represents the continuum of logic flowing from the previous aims (KLF binding to DNA and coupling to epigenetic machinery to regulate colitis) to the current hypothesis. In the process of characterizing the role for EZH2 isoforms at the FOXP3 promoter locus (previous Aim3) we discovered a profound role for EZH2 in FOXP3 repressor function in both murine and human T regulatory cells. This critical and modifiable function within Treg cells in the setting of IBD is the subject of this grant proposal.

Aim 1: Repression of immunoregulatory gene networks by FOXP3 requires the formation of a complex between this transcription factor and EZH2.

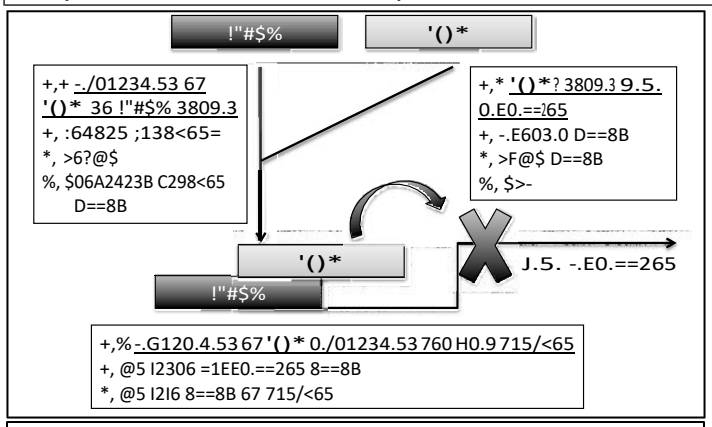


Figure 2 (AIM1): Our model to be tested is that EZH2, FOXP3 complex formation leads to recruitment of EZH2 to target genes, resultant H3K27 trimethylation, gene silencing, and appropriate facilitation of Treg cell function.

Introduction: FOXP3 represses gene networks (4); however the mechanism is not well established. The *objective of this aim* is to define the role for EZH2 in FOXP3-mediated gene repression. We will test the *working hypothesis* that EZH2 is recruited to target genes in complex with FOXP3 (Figure 2). Our *approach* will be domain mutational analyses of EZH2 and FOXP3. We will test the function of these mutations by both molecular (target gene repression) and cellular (Treg suppressor function) assays. The *rationale for this aim* is that understanding the mechanism of FOXP3 repressor function will lead to therapy to enhance Treg cell function *in vivo*. Our *expectation* is to identify the protein domains within EZH2 and FOXP3 required to repress target genes and enhance Treg function. This *knowledge is important* because EZH2 modifying agents (EPZ-6438 and GSK-126) are currently in clinical trials for lymphoma; thus insights derived from this grant into *in vivo* Treg function are significant and impactful.

Justification, feasibility and preliminary data: We have identified a critical role for **EZH2 HMT activity** in mediating **Treg suppressor function**. We have generated a conditional, functional knockout of EZH2 through deletion of the enzymatically active SET domain under the control of the FOXP3 promoter (FOXP3$^{\$EZH2}$). By 35 days, 80% of FOXP3$^{\$EZH2}$ animals succumb to a **lethal inflammatory disorder** similar to the *scurfy* mouse characterized by intense inflammatory infiltrate of multiple organs (survival curve, n=107 total mice, Figure 3A). FOXP3$^{\$EZH2}$ animals surviving to 8 weeks exhibit significantly increased susceptibility to colitis upon exposure to subclinical (1%) DSS

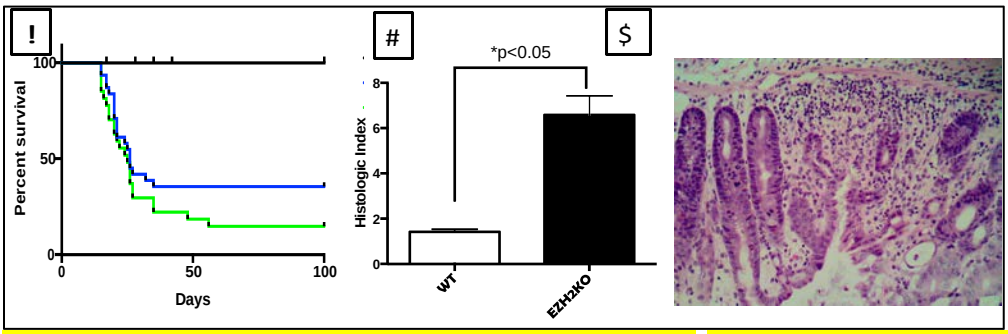


Figure 3: FOXP3$^{!EZH2}$ mouse. (A) Survival curve of WT (black), Heterozygous (blue), and Homozygous (green) littermates for EZH2 deletion. (B, C) Colitis index and representative histology demonstrating severe colitis in 8 week old FOXP3$^{!EZH2}$ mice in response to subclinical (1%) DSS challenge (n=16 total mice).

challenge (histology, n=16 total mice, Figure 3B, C). The histologic activity index represents blinded scoring for 9 criteria as previously published (28). Note lamina propria expansion, goblet cell loss, crypt destruction, inflammatory cell infiltration and ulcer in severely affected FOXP3$^{\$EZH2}$ mice (Figure 3C). FOXP3$^{\$EZH2}$ lymphocytes develop at expected frequency, and upon isolation, the cells express the typical cell surface repertoire of regulatory proteins (Figure 4); however they lack IL10 and produce proinflammatory cytokines including significant upregulation of **IFN! and IL17** (Figure 5A). FOXP3$^{\$EZH2}$ cells **function poorly** *in vitro* (Figure 5B). Furthermore, we discovered a **direct interaction** between EZH2 and FOXP3 by proximity ligation assay (PLA) in the nucleus of WT Treg cells (Figure 6). Thus, we conclude that **FOXP3 and EZH2 co-localize in the nucleus and that Treg specific EZH2 HMT activity is required for normal immune homeostasis and *in vitro* FOXP3+ Treg function.** This discovery is significant as the regulation of this complex through cell signaling networks will be an important modifiable mechanism to target for the treatment of immunological disorders.

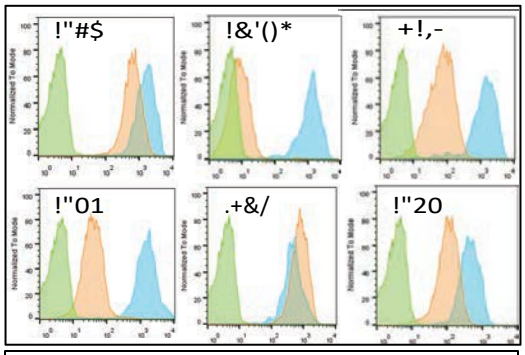


Figure 4: Cell surface staining of FOXP3$^{!EZH2}$ Treg cells (blue histogram) demonstrates upregulation of established immunoregulatory proteins. Marked upregulation over WT Treg cells (red histogram) suggests an activated Treg phenotype. Green histogram represents IgG control (representative of 3 independent experiments).

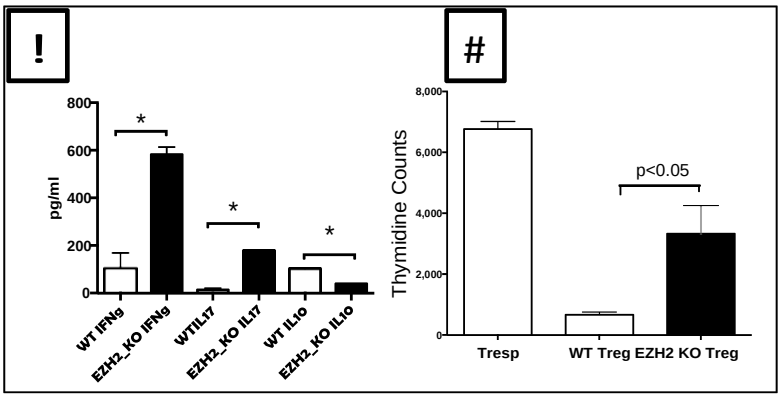


Figure 5: FOXP3$^{!EZH2}$ Treg cells express pro-inflammatory cytokines and function poorly *in vitro*. (A) ELISA of supernatants of FOXP3+ cells. (B) Proliferation assay shows that FOXP3$^{!EZH2}$ Treg cells (closed histogram) fail to suppress WT T responder (WT Tresp, 1:1 ratio) when compared to WT Treg cells (*p<0.05, n=6 mice).

Research Design: The general strategy of Aim 1 is to map interaction domains (1.1) and test domain function through assays of gene repression (1.2) and Treg cell suppressor function (1.3). The laboratory has published experience with co-immunoprecipitation (Co-IP) assays, luciferase reporter systems, ChIP assay, *in vitro* suppression assays, and *in vivo* colitis models (3,20,26). Overexpression of mutant constructs will be performed in cell lines by nucleofection and primary cells by adenoviral transduction. In our published experience, nucleofection efficiency in cell lines approximates 20% and adenoviral transduction efficiency is greater than 75%.

1.1 Biochemical approach to define the FOXP3-EZH2 complex

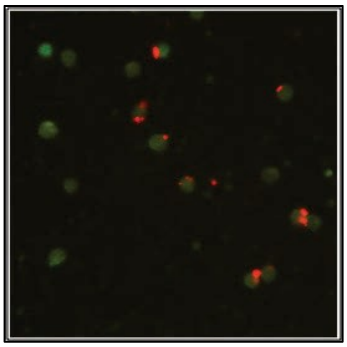


Figure 6: FOXP3 and EZH2 co-localize in primary Treg cells. Proximity ligation assay showing direct interaction of EZH2 and FOXP3 (red punctate staining) in FOXP3+ cells (green nuclei). No hybridization was evident upon addition of ligation mix, probe, and control Ab.

We will determine the interacting domains between EZH2 and FOXP3 using Co-IP with validation by PLA. We will transfect FLAG-EZH2 and HIS-FOXP3 domain mutants in primary cells and T cell lines. The deletion mutations (Figure 7) were selected on the basis of protein interacting domains suggesting direct EZH2, FOXP3 interaction (35-37). Results will be validated by PLA in primary Treg cells (Figure 6). In this assay, antibodies directed to either EZH2 or FOXP3 are tagged with oligonucleotide probes that upon binding in proximity (30-40nm) hybridize to form circular DNA strands (38). DNA circles serve as template to amplify fluorescent probes indicating proximity. We expect EZH2 to complex with FOXP3 in a direct fashion, and the PLA results support this expectation. We expect to identify the specific domains relevant to this interaction. As both EZH2 and FOXP3 have additional functions independent of each other, only with knowledge of precise interaction domains can we separate the biological effects of FOXP3-associated EZH2 function from EZH2 function broadly in lymphocytes.

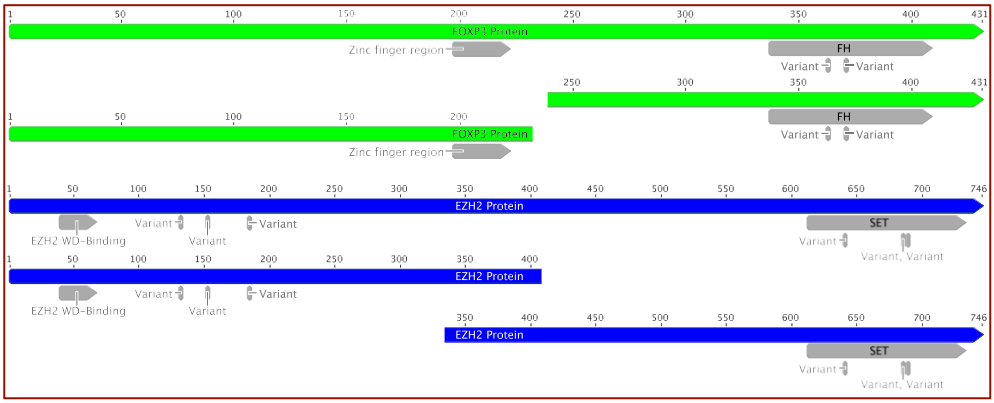


Figure 7: N and C terminal mutant constructs for FOXP3 (green) and EZH2 (blue) proteins. Regions of interest are the Zinc finger and forkhead (FH) domains of FOXP3. Disease inducing variants (human IPEX) are present in the FH domain. Similarly, the WD-binding domain and the enzymatic SET domain are shown in EZH2. Lymphoma associated variants are demonstrated.

1.2 The functional relevance of EZH2 in target gene repression. We have performed RNA-Seq using CD4+ cells isolated from resection specimens in patients with active Crohn's disease and ileal biopsies of healthy age and gender matched control subjects. We analyzed existing ChIP-Seq datasets and have discovered 260 overlapping targets of FOXP3 and EZH2 (39-41). We then compared gene expression of these overlapping targets between samples from patients with CD and controls. From this broad dataset we have chosen RORA (RAR Orphan Receptor A) and IRF4 (Interferon Regulatory Factor-4) as models to study the functional relevance of EZH2 in Treg cells. **RORA and IRF4 are established targets of EZH2** (42,43) and both are significantly upregulated in our Crohn's disease sample (Figure 8). Moreover, both RORA and IRF4 are key transcription factors in the development of Th17 cells, an immune subset responsible for human and murine colitis (44-49). Thus, we will use transcriptional regulation of these genes to test *in vitro* functional relevance of EZH2 and EZH2, FOXP3 complex formation using reporter assays (1.21) and ChIP assay (1.22).

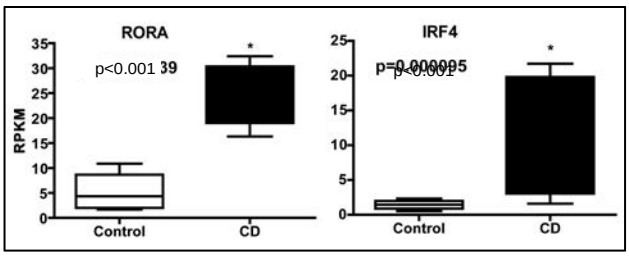


Figure 8: RORA and IRF4 are significantly upregulated in CD4+ lymphocytes isolated from Crohn's lesions. The histograms represent reads per kilobase of transcript per million reads mapped (RPKM) for RORA and IRF4 in RNA-Seq analysis of CD4+ lymphocytes isolated from Crohn's disease resection specimens or ileal biopsies of healthy age, sex match control subjects (n=12 subjects).

1.21 : Luciferase reporter assay: We will use luciferase reporter assays of the RORA and IRF4 promoters in T cell lines. We will nucleofect EZH2 with titrated quantity of FOXP3 into reporter cell lines. We will determine the relevance of FOXP3 to the function of EZH2 through quantitative luciferase assay. We will perform identical assays utilizing EZH2 and FOXP3 domain mutants (Figure 7) to test the requirement of EZH2 and FOXP3 physical interaction for repressor function.

1.22 : ChIP assay: The mechanism of EZH2-mediated silencing is H3K27 trimethylation (H3K27me3) and chromatin compaction at target promoter loci. The presence and function of EZH2 at a particular promoter locus can be assayed using Chromatin Immunoprecipitation (ChIP) for the H3K27me3 histone "mark." Utilizing FOXP3$^{\$EZH2}$ Treg cells, we will transduce WT or EZH2 mutant constructs and perform ChIP assay of the RORA and IRF4 promoter for the presence of H3K27me3 marks, EZH2, and FOXP3. This animal line has been crossed to a mutant transgenic mouse line expressing the coxsackie adenovirus receptor (CAR) to allow efficient gene transduction in resting lymphocytes (26). Essential reagents include FOXP3$^{\$EZH2}$ mouse colony and adenoviral WT or EZH2 mutant constructs (Figure 7). Our laboratory has published experience in ChIP and adenoviral transduction (1,20,26). We expect WT EZH2 but not mutants predicted to disrupt FOXP3-mediated recruitment to repress RORA and IRF4 through deposition of H3K27me3 histone marks.

1.3 The *in vitro* and *in vivo* functional relevance of EZH2 target gene repression.

Finally, we will test the significance of EZH2 target gene repression on Treg suppressor function *in vitro* (1.31) and *in vivo* (1.32).

1.31 : The *in vitro* assay consists of co-culture of naïve FOXP3= CD4+ T cells (T responder cells) with titrated amounts of Treg cells in the presence of conditions to stimulate T responder cell proliferation (anti-CD3, anti-CD28) (1). We will transduce FOXP3$^{\$EZH2}$ Treg cells with WT EZH2 and EZH2 mutant constructs (Figure 7) to test dependence of *in vitro* suppressor function upon EZH2 and FOXP3 complex formation.

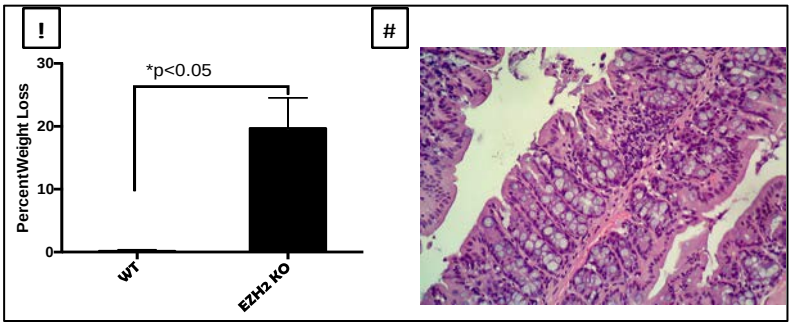


Figure 9: FOXP3$^{!EZH2}$ Treg cells do not suppress Rb^{hi} mediated colitis. Recipient mice (n=4) of mutant Treg cells lost more weight (A, black histogram) and demonstrated severe colitis (B, representative histology from Rb^{hi} transfer) when compared to recipients of WT Treg cells (n=4). Note goblet cell loss, lamina propria expansion and intense inflammatory cell infiltration.

1.32 *In vivo* colitis assay: We will test *in vivo* function of FOXP3$^{!EZH2}$ Treg cells in colitis prevention. We will utilize the CD45^{Rbhi} transfer model of

colitis, a model in which we have extensive experience, mimics well the phenotype of human IBD, and in which preclinical trials have translated well to human therapy (50-52). For the *in vivo* model, 350,000 naïve CD4+ lymphocytes are transferred into immunodeficient RAG^{null} mice alone, or along with 100,000 FOXP3+ Treg cells. Our experience with this system is well published (52-55). We have established that FOXP3^{EZH2} Treg cells do not prevent colitis upon co-transfer with WT naïve CD4+ cells (Figure 9). We have developed a new model of inducible EZH2 deletion through a tamoxifen-inducible CRE-mediated recombination system driven by the chicken beta actin promoter/enhancer. Upon cross with the EZH2 flox mouse, EZH2 deletion can be obtained in any mouse cell upon treatment with tamoxifen. We harvested Treg cells from this mutant animal (and WT control), and co-transferred mutant (or WT) Treg cells along with WT CD45Rb^{hi} cells into RAG^{null} mice. All animals were treated with a 5-day tamoxifen injection protocol. At 6 weeks, RAG^{null} mice injected with mutant Treg cells exhibited extreme weight loss and colitis while RAG^{null} mice treated with control Treg cells appeared healthy (Figure 9). We will now transduce WT EZH2 or mutant constructs into FOXP3^{EZH2} Treg cells to test the requirement for EZH2 to associate with FOXP3 for the prevention of murine IBD. Colitis severity will be assessed by disease activity index (DAI), blinded histology assessment, cytokine expression profile of T cells isolated from mesenteric lymph nodes, and cytokine assessment of colonic supernatants (28). We expect adenoviral delivery of the WT EZH2 to rescue *in vivo* function of FOXP3^{EZH2} Treg cells. Furthermore, we expect that EZH2 domain mutants, disrupted in their capacity to complex with FOXP3 will not prevent colitis. This finding would indicate the critical nature of this complex to prevent inflammation.

Anticipated Results, potential pitfalls, and alternative approaches: The objective of this aim is to define the role for EZH2 in FOXP3-induced gene repression and subsequent Treg function. We expect EZH2 to complex with FOXP3 in a direct fashion (1.1). We expect WT EZH2 to repress RORA and IRF4, but only in the presence of FOXP3 (1.2). We expect the functional relevance of the EZH2 and FOXP3 interaction to be *in vitro* suppression of T responder cells and colitis prevention (1.3). These exciting experiments will be the first to test a modifiable epigenetic mechanism in the treatment of IBD.

The first alternative hypothesis considered is an indirect rather than direct EZH2, FOXP3 interaction through obligate EZH2 binding partners EED and Suz12 (35,36). To directly test this possibility we could perform siRNA knockdown of EED and Suz12, and repeat Co-IP assay for WT EZH2 and FOXP3. A second alternative hypothesis is that rather than a requirement for FOXP3, EZH2 utilizes additional mechanisms for target gene localization. Non coding RNAs are increasingly recognized to directly bind with chromatin modifying complexes and play a role in altered gene expression in many tissues and particularly cancer (56). Should EZH2 not require FOXP3 for target gene localization we will perform cross-linking and immunoprecipitation methodology combined with RNAseq to identify EZH2-interacting RNA. Functional knockdown of long noncoding RNA could reveal this potential function.

Aim 2: Inflammatory stimuli, such as IL6 lead to EZH2 phosphorylation and thereby disrupt the enzymatic activity of this epigenomic regulator.

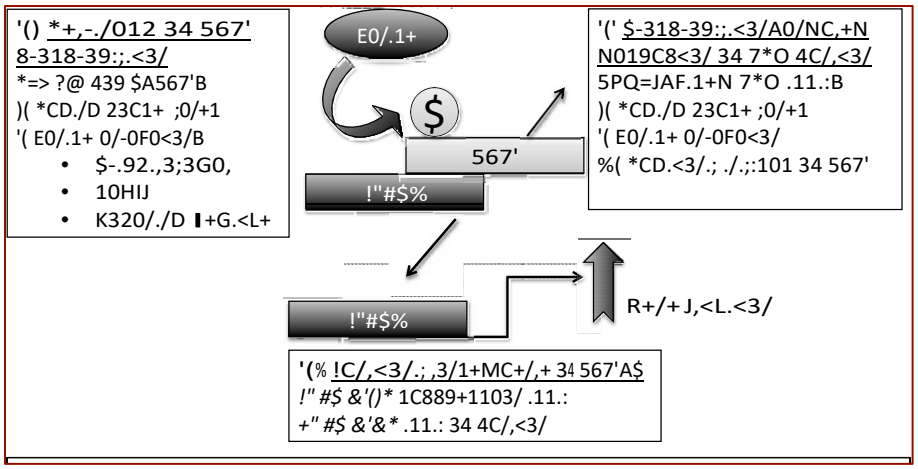


Figure 10 (AIM2): Our model is that a kinase (JAK2) downstream of an inflammatory signal (IL6) modifies EZH2 (Y641 phosphorylation) resulting in impaired HMT activity at FOXP3 target genes (RORA, IRF4) and subsequent Treg cell dysfunction. We will use mutant mouse/cell lines and kinase inhibition to identify the mechanism(s) of EZH2 phosphorylation (by Mass Spec, MS and WB) and resultant HMT function (HMT assay and *in vitro/vivo* assays of suppressor function).

Introduction: In CD4+ T cells, inflammatory signals such as IL1", TNF#, and IL6 abrogate Treg cell development and function (2,16,57-61), while IL10 in a paracrine fashion enhances Treg function (62). Indeed in support of our hypothesis, the requirement for EZH2 HMT activity to the maintenance of Treg cellular phenotype and the conversion of Treg cells to IL17-like T effector cells through IL6 signaling have both recently been reported (6,63); **yet the precise mechanisms governing this cellular conversion are lacking.** The *objective of this aim* is to identify the mechanism(s) by which cytokine signaling pathways regulate EZH2 function in Treg cells using IL6 as the relevant model system. We will test the *working hypothesis* that IL6 signaling modulates Treg function through regulation of EZH2 HMT activity by phosphorylation. Our *approach* is an experimental pipeline that consists of identifying potential posttranslational modifications through bioinformatics with confirmation from MS experiments, protein structure analyses using molecular modeling with validation through mutational screens, and functional assays to definitively test the hypothesis of Aim 2. The rationale for this aim is that we will fill a critical gap in our understanding of inflammation mediated modulation of Treg function. The acquisition of such knowledge is crucial to the development of improved cell based strategies for human IBD based upon *ex vivo* manipulation of Treg responsiveness to inflammatory signals. We *expect* that the disruptive IL6 signaling pathway will entail phosphorylation of EZH2 by JAK2 inhibiting EZH2 enzymatic function. This finding is important, because it will allow for the first time, the development of novel approaches to Treg based therapy in IBD.

Justification, feasibility, and preliminary data: IL6 signaling disrupts Treg cell function. Previous work identifying the disruptive role of IL6 to Treg function has focused on an effect on T responder cells (2). We demonstrate that disrupted function can be dependent upon Treg cell intrinsic IL6 signaling. We utilized a mutant mouse strain with a spontaneous point mutation in the gene encoding the IL6R# resulting in a complete lack of protein (in collaboration with Dr. Chandrashekhar Pasare, UTSW, see letter). Using IL6R-deficient T responder cells and WT Treg cells, we are able to isolate the effect of IL6 signaling to Treg cells in the suppression assay. In Figure 11A, we

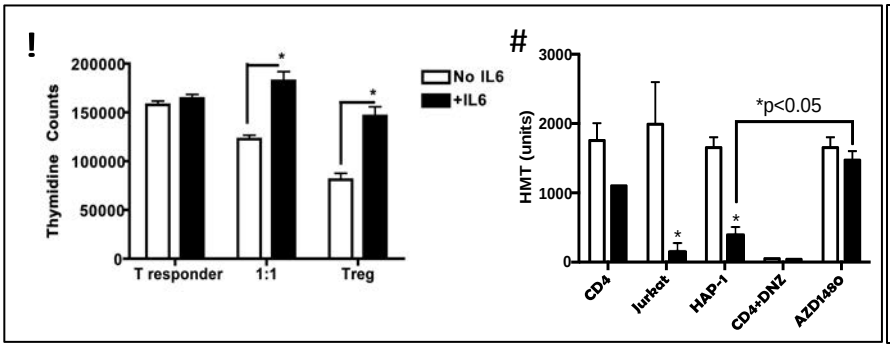


Figure 11: (A) WT Treg cells were plated alone (Treg) or 1:1 with Tresp cells. The Tresp cells were from the IL6R mt mouse. Closed histograms show failure of WT Treg cells to suppress Tresp in the presence of 10ng IL6 (B) Treatment with IL6 (closed histograms) in primary T cells and cell lines inhibits HMT activity. HMT inhibition with 3-deazaneplanocin (DNZ) serves as a control. Inhibition of HMT activity by IL6 is completely abrogated upon treatment with the JAK2 inhibitor AZD1480 (*p<0.05).

demonstrate significant disruption of the *in vitro* suppression assay attributed to IL6 signaling uniquely in Treg cells. Using an ELISA based assay specific for EZH2 (H3K27) HMT activity, we discovered that **IL6 signaling significantly represses EZH2 HMT** activity in both primary WT CD4+ lymphocytes and cell lines (Figure 11B). This repressive effect was only **consistently inhibited by specific inhibition of JAK2**, and only minimally affected by AKT or MAPK pharmacologic inhibition (Figure 11B, AZD1480 and data not shown). To address potential phosphorylation sites attributable to IL6, we performed a **bioinformatic analysis** to predict posttranslational modification sites within EZH2 by compiling and statistically scoring results obtained using NetPhosk 2.0 (64), PhosphoSVM

Seq# pos	NetPhos2.0		PhosphoSVM		GPS3.0	
	Context	*Score*	*Score*	*Kinase*	*Score*	*Kinase*
610-S	IQRGSKKHL	0.992	0.75	MAPK	8	MAPK
639-S	NEFISEYCG	0.942	0.75	MAPK	10	MAPK
664-S	KYMCSFLFN	0.002	0.62	MAPK	3	MAPK
641-Y	FISEYCGEI	0.014	0.53	JAK2	1	JAK2
658-Y	RGKVYDKYM	0.037	0.53	JAK2	7	JAK2
726-Y	LFFDYRYSQ	0.026	0.53	JAK2	3	JAK2

Figure 12: Results of linear motif analysis of EZH2 for predicted phosphorylation sites. The score represents the potential for phosphorylation. The higher the value, the more potential the residue is phosphorylated. Predicted site-specific kinase is listed in column right.

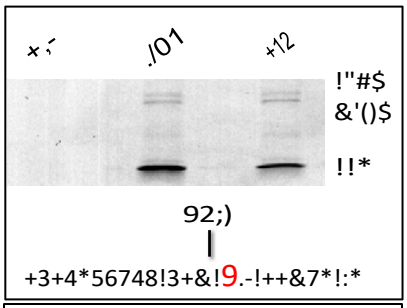
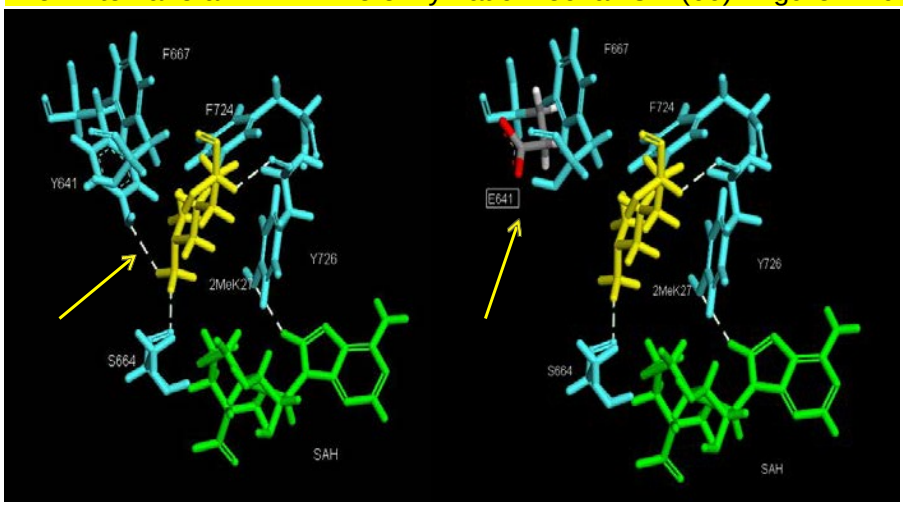


Figure 13: IP of EZH2 and complex partners Suz12 and EED (top panel). MS analysis of lysates obtained from cells treated with IL6 confirmed phosphorylation of Y641.

(65), and GPS 3.0 (66). We focused on putative phosphorylation sites within the enzymatically active SET domain (AA 605-729), as they should have clear impact on the function of this HMT. The demonstrated motifs are predicted **MAPK and JAK2 phosphorylation sites** (Figure 12) and **confirmed by MS to be phosphorylated** upon pull down of native EZH2 (Figure 13). One of the key residues modified by phosphorylation is Y641, which mutations have previously shown to alter human B cell function (67). We gained useful insight into how Y641 phosphorylation may affect the activity of EZH2 through molecular modeling. Briefly, we developed a homology-based model using the SET domain of EZH2, which had been previously crystallized in the absence of both the SAM cofactors and the target histone peptide. These two molecules, which are key for the full HMT activity were obtained by homology superimposition with the SET domain of EHMT1/G9a and SETD2; for both are known to have an EZH2-like enzymatic mechanism (60). Figure 14 demonstrates the modeled structure of the EZH2 methyltransferase domain. Note Y641 engages in a hydrogen bond with the de-protonated $-Nitrogen of the dimethylated lysine that will be tri-methylated, (dash line and yellow arrow, Figure 14 left panel). Using **in silico mutagenesis**, we investigated the potential effects of phosphorylation, by using the well-characterized phosphomimetics (Y to E) and non-phosphorylatable mutations (Y to F) methodology. We find that the **Y641E mutation disrupts the aforementioned hydrogen bond** (Figure 14, right panel). Combined these studies suggest that **phosphorylation of this Y residue may regulate the histone methyltransferase activity of EZH2.** We next performed biochemical experiments to test the validity of these data. We developed a novel system based on using a **CRISPR/CAS-mediated EZH2KO cell** in which the enzymatic activity of transfected WT EZH2 was directly compared with both the phosphomimetic (Y641E) and nonphosphorylated (Y641F) Y641 mutants. As shown in Figure 15, the phosphomimetic mutant **Y641E is inactive** when compared to WT or the non-phosphorylatable Y641F mutant. Thus, given this strong preliminary data, in Aim 2 we will confirm the key modifications relevant to cytokine signaling and test the functional relevance of the biochemical assays through cellular assays and gene expression.



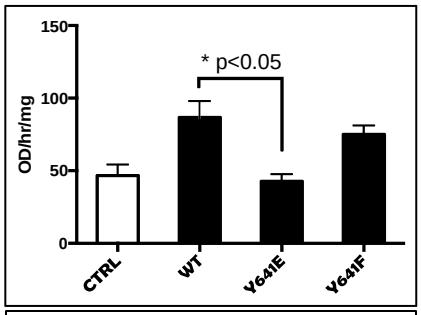


Figure 15: Using ELISA, we show reduction in HMT activity upon mutation of Y641 to the phosphomimetic, Y641E.

Research Design:

The strategy of Aim 2 is to identify functionally relevant post-translational modifications of EZH2 downstream of IL6 through a mass spectrophotometry screen (2.1) followed by a point mutational analysis consisting of functional assays of enzymatic activity (2.2), gene regulation and Treg suppressor function (2.3). We use Y641 as a powerful example of feasibility and significance; however we will systematically assess all 6 phosphorylation sites of the SET domain identified by the mass spectrophotometry screen.

2.1 Establish the mechanism of modifications to the EZH2 SET domain.

The preliminary data (bioinformatic analysis and pharmacologic inhibitor screens) are supportive of the substantial role for **JAK2** in regulation of EZH2 enzymatic activity. To confirm this mechanism, we will stimulate primary Treg cells and cell lines (Jurkat and Hap-1) with IL6 (10ng/ml) in the

presence of JAK2 modulation and measure phosphorylation of the EZH2 SET domain by mass spectrophotometry and western blot. The rationale for both outcome assessments is the enhanced data acquisition of MS balanced with the speed and low cost of western blot analysis. We have developed and optimized a rabbit polyclonal Phospho-Y641 antibody.

We will inhibit JAK2 in three ways: pharmacologic JAK2 inhibition (AZD1480), JAK2 siRNA treatment, and overexpression of a JAK2 dominant negative construct (Y792F) (68). Constitutively active JAK2 V617F (69) will be used as an important gain-of-function control. We will use primary cells from our recently developed FOXP3-specific, IL6R conditional KO ($FOXP3^{\Delta IL6R\alpha}$) as a relevant negative control. We expect IL6 to lead to phosphorylation of EZH2 and subsequent impairment of HMT activity through activation of JAK2. Furthermore, we expect the $FOXP3^{\Delta IL6R\alpha}$ Treg cells to be resistant to EZH2 phosphorylation.

2.2 Test the functional significance of EZH2 SET domain phosphorylation.

IL6 signaling leads to both EZH2 phosphorylation and reduced HMT activity in our system (Figure 11 and 13); thus we will now establish causality of impairment of EZH2 HMT activity to EZH2 phosphorylation. The outcome variable for the subsequent experimental set is IL6-induced impairment of HMT activity in nuclear extracts of test cells. We have generated a library of EZH2 mutant constructs designed to mimic (S to D; Y to E) or prevent (S to A; Y to F) phosphorylation. We have established an ideal *in vitro* cell system in which to screen the HMT activity of EZH2 mutant constructs. Utilizing a haploid fibroblast cell line (HAP-1) (70) in which the CAS9 system has induced a frameshift mutation in exon 7 of human EZH2, we demonstrate significant reduction of HMT activity in the Y641E mutant (mimic phosphorylation) when compared to WT EZH2 control (Figure 15). We have produced our first candidate mutant (Y641E) adenovirus, which will be used to confirm functional relevance in primary T cells.

HMT assay will be run using nuclear extracts from $FOXP3^{\Delta EZH2}$ Treg cells upon adenoviral transduction of either WT-EZH2 or mutant EZH2. We expect that Y641F (unable to be phosphorylated and widely noted to be hyperactive in human lymphoproliferative conditions) will function well in the presence of IL6 as measured by HMT activity. Conversely, we anticipate, the Y641E mutant to have an impaired HMT activity when compared to the WT construct due to the phosphomimetic properties of the glutamic acid substitution. Our team has extensive experience in mutational analyses to identify modifiable residues (20,71).

2.3 Establish the functional relevance of SET P-Y641-EZH2 within the whole cell system.

IL6 stimulation reduces HMT activity in nuclear extracts of primary lymphocytes (Figure 11B). Thus in this subaim we will extend our preliminary findings to whole cell assays of functional relevance including: 2.31) luciferase reporter and 2.32) *in vitro* suppression assays.

2.31 : Luciferase reporter assays using genomically integrated T cell lines. As we have done previously, we generated genomically integrated T cell lines to study EZH2-dependent events in a non-episomal system (3,20). Using the Jurkat FLP system we created two reporter cell lines with the RORA and IRF4 promoters genomically integrated in frame with a luciferase reporter. RORA and IRF4 are ideal targets as both are established targets of EZH2 (42,43) and upregulated in CD (Figure 8). Using this system we will overexpress WT EZH2 or EZH2 Y641 mutants to test the dependence of IL6 induction of RORA/IRF4 on EZH2 Y641 phosphorylation.

2.32 : *In vitro* suppression assays. We will test the capacity of WT and mutant EZH2 constructs to rescue *in vitro* suppressor function in the setting of IL6. Utilizing adenoviral transduction of $FOXP3^{\Delta EZH2}$ Treg cells, we will introduce WT or EZH2 Y641 mutant constructs and perform suppression assays in the presence or absence of IL6. As EZH2 Y641E mimics phosphorylation downstream of IL6 signaling, we expect the Y641E mutant to be a loss of function mutation, even without IL6 stimulation. Loss of function would be evident in the de-repression of luciferase expression and the inability to suppress T responder cell proliferation. We expect the EZH2 Y641F mutant (unable to be phosphorylated) to be a gain of function mutation, evident in the presence of IL6. Gain of function in this context will be demonstrated through the repression of luciferase and retained capacity to suppress in the setting of IL6 stimulation.

Anticipated Results, potential pitfalls, and alternative approaches: We expect IL6 stimulation to lead to phosphorylation of EZH2 through activation of JAK2 (2.1), and phosphorylation to lead to inactivation of HMT activity (2.2). We expect this disruption to be evident in the transcriptional activation of IRF4 and RORA (2.31) and impairment of Treg *in vitro* function (2.32). We consider here several alternative hypotheses. First, we recognize additional inflammatory pathways including the TNF superfamily receptors OX40, 4-1BB, TNFR1 and IL1 family receptors have been linked to Treg dysfunction (72). The experimental pipeline (MS screen and mutational analysis) is amenable to any cytokine signaling input and could be used in future mechanistic studies of cytokine regulation of EZH2. Furthermore, IL10 has been suggested to enhance Treg function; however the mechanism of this effect is not well understood. We could explore the effect of IL10 on alternative post-translational modifications and subsequent effect on EZH2 HMT activity. Secondly, while IL6 stimulation clearly leads to SET domain phosphorylation there are other potentially important modifications and mechanisms to disrupt EZH2 HMT activity. Phosphorylation of EZH2 may lead to alternative substrate specificity and complex formation for this HMT. In glioblastoma cell lines, EZH2 methylates Stat3 at the K180 position, and this methylation activity is enhanced through EZH2 serine phosphorylation at S21 by AKT (11). To investigate this possible alternative outcome, we could isolate the EZH2 complex upon treatment with IL6 to identify alternative binding partners and substrates. Our collaborative team has extensive experience in the isolation and characterization of HMT complexes should this be required (73).

Aim 3: Inhibition of the IL6 to EZH2 signaling pathway permits sustained Treg suppressive function in intestinal inflammation.

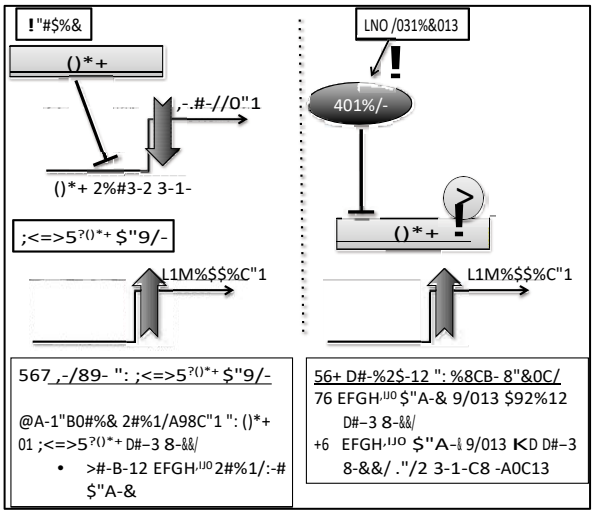


Figure 16 (AIM3): Our model is that Treg specific dysfunction of EZH2 (either by genetic deletion or IL6 induced phosphorylation) leads to colitis. We will test this model through adoptive transfer of WT and mutant Treg cells into the FOXP3$^{\Delta EZH2}$ mouse. Furthermore, we will test the capacity for Treg cells mutant in the IL6 signaling pathway or the EZH2 protein to function in the setting of active colitis through TREATMENT rather than prevention studies.

Introduction: It is established that FOXP3+ Treg cells **prevent** colitis more efficiently than they **treat** active colitis in animal model systems (74-76). Furthermore, loss of regulatory or even gain of pro-inflammatory function of FOXP3+ cells in human IBD is evident given the frequency and cytokine expression pattern of FOXP3+ cells in actively inflamed mucosa of IBD patients (1,77). The *objective of this aim* is to perform a pre-clinical animal trial of Treg cells engineered to function in the inflamed intestine. We will test the working hypothesis that sustained EZH2 HMT activity in Treg cells permits Treg cellular function in the setting of active inflammation. Our *approach* will be *in vivo* assays of Treg suppression in the aforementioned T cell transfer model of colitis. The *rationale for this aim* is that we will fill a gap in knowledge, without which we cannot understand the mechanism for sustained Treg function in the setting of active inflammation. This knowledge is critical to the development of improved cell based strategies for human IBD. When the proposed studies for Aim 3 have been completed, it is our *expectation* that disruption of the IL6 to EZH2 signaling pathway in Treg cells will lead to successful treatment of active colitis. Such a finding would be of importance, because current non-targeted, systemic anti-IL6 therapy has a poor safety profile related to impairment of epithelial cell homeostasis (78,79).

Justification, feasibility and preliminary data: Antibody blockade of the IL6R is effective in adult Rheumatoid Arthritis (RA) (80) and has been used in very early onset IBD (81). Similarly, inhibition of Jak1/3 is effective in adult RA (82) and is undergoing advanced clinical trials in IBD (83). Infrequent occurrences of intestinal perforation in RA patients suggest a requirement for cell subtype specific therapy over the pan-inhibition of this pleiotropic cytokine (78,79). In this aim, we look to inhibit this pathway uniquely in Treg cells *in vivo* to treat active colitis. Through RNA-Seq methodology

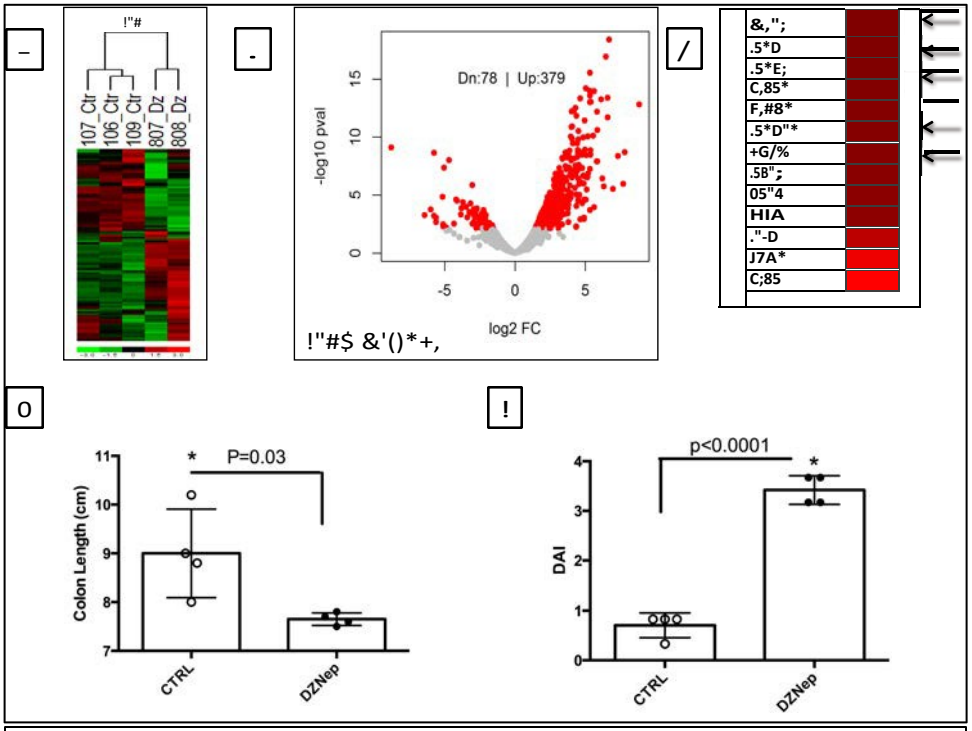


Figure 17: EZH2 controls IL6 inflammatory pathways. (A) Heat map of RNAseq of CD4+ cells isolated from Crohn's lesions. The heat map is the IL6 pathway and shows marked variance between control subjects (CTR) and disease (Dz). (B) Volcano plot of relative expression of EZH2 targets comparing DZ to CTR. Red dots show significant variance. (C) Heat map of microarray from FOXP3$^{\uparrow EZH2}$ Treg cells vs control. Red cells show upregulation of genes. Note (arrow) genes of the Th17 and Th1 pathway. (D and E) 2% DSS colitis in WT mice treated with the EZH2 inhibitor DZNep (1mg/kg) at Day 2 and 5. Colon length and DAI show severe colitis upon EZH2 inhibition (n=8).

using CD4+ T cells isolated from Crohn's lesions, we have identified marked upregulation of IL6 induced signaling networks (Figure 17A). IL6 signaling leads to phosphorylation of EZH2 and impaired HMT activity. Thus, it is important to note significant variation in the expression of EZH2 targets between health and disease in the same cells (Figure 17B). Furthermore, the significant majority (379/457 target genes) is upregulated, a finding suggestive of impaired EZH2 HMT function (Figure 17B). Congruent with these observations, microarray data obtained from FOXP3$^{\$EZH2}$ Treg cells demonstrate marked upregulation of proinflammatory pathways regulated by IL6 signaling (Figure 17C). Finally, we demonstrate that inhibition of EZH2 (DZNep 1mg/kg ip) HMT activity significantly exacerbates DSS colitis (Figure 17D, E). Thus, we have observed IL6 to inhibit EZH2, a protein that itself is a negative regulator of IL6-induced proinflammatory pathways relevant to human IBD. Moreover, pharmacologic inhibition of EZH2 (mimicking the IL6 effect) worsens DSS induced colitis. We now propose to treat active colitis through genetically engineered Treg cells. Our model system will be the CD45Rb^{hi} transfer model of colitis (discussed in Aim 1 and demonstrated in Figure 9), and Treg cell therapy will be applied to treat rather than prevent active inflammation. We have recently utilized this precise methodology to demonstrate the enhanced function of murine Treg cells engineered to maintain an active FOXP3 promoter locus in the inflammatory setting (84). Focusing now on the IL6 to EZH2 pathway, we will perform identical experiments (genetic manipulation of Treg cells and co-transfer in the Rb^{hi} mediated model of colitis) to utilize Treg cellular therapy in active colitis.

Research Design:

The general strategy of Aim 3 is to utilize adoptive transfer systems and genetically engineered Treg cells to demonstrate the capacity for Treg cells to function in the inflamed intestine and treat active colitis. We will use the naïve T cell into RAG^{null} colitis model as our model system, and we will genetically engineer Treg cells using adenoviral transduction (3.1) and TALEN methodology (3.2). Our efficient use of adenoviral constructs has been addressed above, and our genetic editing will be performed in collaboration with the Genetics and Model Systems Core with the Center for GI Signaling, Mayo Clinic (Dr. Stephen Ekker, see letter of collaboration).

3.1 Treatment of active colitis with EZH2 mutant cell lines.

We have demonstrated loss of Treg suppressor function *in vitro* upon treatment of co-culture assays with IL6. We now will test the effect of therapy directed to this pathway through *in vivo* assays of Treg function. We will use the IL6R"-deficient mice as the donor of Treg cells. We will harvest Treg cells from the FOXP3$^{\$IL6R"}$ mouse line and test the *in vivo* regulatory capacity of these cells to treat

colitis. As per our published data (84), we will inject titrations of 100-300,000 cells of WT or IL6R" mutant Treg cells into recipient RAG^{null} animals with established colitis (4 weeks post T effector cell transfer). We expect IL6R" mutant cells to robustly treat colitis, as compared to WT Treg cells. We will rescue FOXP3$^{\$EZH2}$ Treg cells with EZH2 WT, Y641E, or Y641F mutant constructs. We expect the EZH2 Y641F (gain of function) but not the EZH2 Y641E (loss of function) to effectively treat established colitis.

3.2 Treatment of active colitis with genetically edited cells.

Finally, we will test *ex vivo* cellular therapy with clear translational potential to human IBD. Our department of Biochemistry and Molecular Biology has extensive experience with transcription activator-like effector nucleases (TALENs) and has recently published successful genetic editing of primary T cells (85,86). We will isolate WT Treg cells from C57/BL6 mice and perform genetic editing/deletion of IL6R" using TALENs (see letter of collaboration). As per previous study, 20 mcg of TALEN construct (left arm and right arm) and pEGFP-N1 (Clontech, Mountain View, CA, USA) DNAs will be transiently nucleofected by electroporation into primary Treg cells (RFP+). Forty-eight hours after nucleofection, GFP-expressing cells will be selected by fluorescence-activated cell sorting. Deletion of the IL6R" will be confirmed by flow cytometry. The TALEN edited IL6R" null cells will be expanded using Treg *ex vivo* cell expansion methods we have optimized (87), and injected into recipient RAG^{null} animals with established colitis as above. We expect genetic editing *ex vivo* of WT Treg cells to enhance suppressor function in the setting of active colitis, and this finding to thus provide a clear roadmap for translational cell therapy studies in patients with IBD.

Anticipated Results, potential pitfalls, and alternative approaches: We expect in 3.1 robust treatment of active colitis with FOXP3+ Treg cells impaired in IL6R to EZH2 signaling either at the level of the receptor (IL6R" KO Treg cells) or EZH2 (EZH2 Y641F mutant) when compared to WT Treg cells. We expect in 3.2 genetic editing *ex vivo* of WT Treg cells to enhance suppressor function in the setting of active colitis, and this finding to provide pre-clinical data for translational cell therapy studies in patients with IBD. As the Y641F mutant in *whole lymphocyte populations* has been associated with lymphoma, this particular mutation *uniquely within the Treg subset* will require extensive analysis. To test the long-term behavior of mutant Treg cells, we could perform rescue experiments of the scurfy mouse (no functional Treg cells) to study function and toxicity. A second alternative hypothesis is that impaired Treg function in the setting of inflammation results not from intrinsic Treg dysfunction but the resistance to Treg suppressive mechanisms by T effector cells. This hypothesis is readily testable using our current reagents through the treatment of colitis induced by IL6R" mutant T effector cells with WT Treg cells. We can now dissect the role for EZH2 in any cell population using tamoxifen-inducible Cre-ER(T) mutant mouse under the promotional control of actin. A third alternative hypothesis is that EZH1, not EZH2 is the primary target of IL6 signaling in Treg cells. Beyond initiation of the H3K27me3 mark (EZH2-containing Polycomb Repressor Complex 2 function), maintenance of the mark by the EZH1 HMT may well be involved in maintained Treg function. Given the strength of the preliminary data and the phenotype of the conditional EZH2 KO mouse, we have necessarily chosen to focus on EZH2; however our laboratory and collaborative partners have the necessary tools and experience to dissect additional HMT pathways should these investigations become necessary.

Conclusion: We are studying an innovative membrane to nucleus signaling pathway connecting environmental inflammatory signals (IL6R) to cell differentiation machinery (EZH2) responsible for Treg cell fate and function. Our work has clear biomedical relevance to patients with IBD. Moreover, our work represents a continuum beginning with basic mechanisms of kinase regulation of EZH2 (this proposal) to the *ex vivo* manipulation of Treg cells for cell therapy of IBD. The work is significant as it should lead to first-in-man studies of engineered Treg cells in human IBD.

Aims	Year 1	Year 2	Year 3	Year 4	Year 5
SA1					
SA2					
SA3					

PROGRESS REPORT PUBLICATION LIST:

AI89714 Competitive Renewal Publication List:
Original research publications emanating directly from experiments designed in previous aims:

1. Xiong, Y., Khanna, S., Grzenda, A.L., Sarmento, O.F., Svingen, P.A., Lomberk, G.A., Urrutia, R.A., Faubion, W.A., Jr. (2012). Polycomb antagonizes p300/CREB-binding protein-associated factor to silence FOXP3 in a Kruppel-like factor-dependent manner. Journal of Biological Chemisry, 287(41):34372-85. PMCID: PMC3464543. (AIM1 and AIM3)

2. Xiong, Y., Svingen, P.A., Sarmento, O.O., Smyrk, T.C., Dave, M., Khanna, S., Lomberk, G.A., Urrutia, R.A., Faubion, W.A., Jr .(2014) Differential coupling of KLF10 to Sin3-HDAC and PCAF regulates the inducibility of the FOXP3 gene. American Journal of Physiology Regulatory, Integrative and Comparative Physiology, 307(6):R608-20. PMCID: PMC4166759 (AIM1)

3. Papadakis, K.A., Krempski, J., Reiter, J., Svingen, P., Xiong, Y., Sarmento, O.F., Huseby, A., Johnson, A.J., Lomberk, G.A., Urrutia, R.A., Faubion, W.A.. (2015) Krüppel-like factor KLF10 regulates transforming growth factor receptor II expression and TGF-β signaling in CD8+ T lymphocytes, American Journal of Physiology Cell Physiology, 308(5):C362-71. PMCID: PMC4346734

4. Sarmento, O.F., Svingen, P.A., Xiong, Y., Xavier, R.J., McGovern, D., Smyrk, T.C., Papadakis, K.A., Urrutia, R.A., Faubion, W.A..(2015). A novel role for KLF14 in T regulatory cell differentiation. Cellular and Molecular Gastroenterology and Hepatology, 1(2):188-202. PMCID: PMC4349492.

5. Mathison, A., Grzenda, A., Lomberk, G., Velez, G., Buttar, N., Tietz, P., Hendrickson, H., Liebl, A., Xiong, Y.Y., Gores, G., Fernandez-Zapico, M., Larusso, N.F., Faubion, W. , Shah, V.H., Urrutia, R.. (2013) Role for Kruppel-like transcription factor 11 in mesenchymal cell function and fibrosis. PLoS One. 8(9):e75311. PMCID: PMC3775729. (AIM2)

6. Okou, D.T., Mondal, K., Faubion, W.A. , Kobrynski, L.J., Denson, L.A., Mulle, J.G., Ramachandran, D., Xiong, Y.,Svingen, P., Patel, V., Bose, P., Waters, J.P., Prahalad, S., Cutler, D.J., Zwick, M.E., Kugathasan, S. (2014) Exome Sequencing Identifies a Novel FOXP3 Mutation in a 2-Generation Family With Inflammatory Bowel Disease. Journal of Pediatric Gastroenterology and Nutrition, 58(5):561-8. PMCID: 4277865. (AIM2)

7. Rahman, M.K., Offord, C.P., Khanna, S., Ford, G.C., Perrson, X.M., Svingen, P.A., Xiong, Y., Bajzer, Z., Faubion, W.A. .(2013) Regulatory T cell kinetics in the peripheral blood of patients with Crohn's disease. Human Immunology, 74(2):145-50. PMCID: PMC4224946 (AIM2)

8. Grzenda, A., Lomberk, G., Svingen, P., Mathison, A., Calvo, E., Iovanna, J., Xiong, Y., Faubion, W. , Urrutia, R. (2013) Functional characterization of EZH2beta reveals the increased complexity of EZH2 isoforms involved in the regulation of mammalian gene expression. Epigenetics and Chromatin, 6(1):3. PMCID: PMC3606351. (AIM3)

9. Mills, L.D., Zhang, L., Marler, R., Svingen, P., Fernandez-Barrena, M.G., Dave, M., Bamlet, W., McWilliams, R.R., Petersen, G.M., Faubion, W, Fernandez-Zapico, M.E.. (2014) Inactivation ofthe Transcription Factor GLI1 Accelerates Pancreatic Cancer Progression. Journal of Biological Chemistry, 289(23):16516-25. PMCID: PMC4047418. (AIM1 and AIM3)

10. Dave, M., Hayashi, Y., Gajdos, G.B., Smyrk, T.C., Svingen, P.A., Kvasha, S.M., Lorincz, A., Dong, H., *Faubion, W.A., Jr, *Ordog, T. (2015) Stem cells for murine interstitial cells of cajal suppress cellular immunity and colitis via prostaglandin e2 secretion. Gastroenterology,148(5):978-90. PMCID: PMC4409492 (* Co-corresponding authors)

Collaborative projects not directly related to prior aims:

1. Li, H., Chan, L., Bartuzi, P., Melton, S.D,, Weber, A., Ben-Shlomo, S., Varol ,C., Raetz, M., Mao, X., Starokadomskyy, P., van Sommeren, S., Mokadem, M., Schneider, H., Weisberg, R., Westra, H.J.,

Esko, T., Metspalu, A., Kumar, V., Faubion, W.A., Yarovinsky, F., Hofker, M.,Wijmenga, C., Kracht, M, Franke, L., Aguirre, V., Weersma, R.K., Gluck, N., van de Sluis, B.,Burstein, E.(2014). Copper metabolism domain-containing 1 represses genes that promote inflammation and protects mice from colitis and colitis-associated cancer. Gastroenterology, 147(1):184-195.e3.
Epub 2014 Apr 13. PubMed PMID: 24727021; PubMed Central PMCID: PMC4086320.

2. Kauder, S.E., Santell, L., Mai, E., Wright, L.Y., Luis, E., N'Diaye, E.N., Lutman, J., Ratti, N., Sa, S.M., Maun, H.R., Stefanich, E., Gonzalez, L.C., Graham, R.R., Diehl, L., Faubion, W.A.. Jr., Keir, M.E., Young, J., Chaudhuri, A., Lazarus, R.A., Egen, J.G. (2013). Functional consequences of the macrophage stimulating protein 689C inflammatory bowel disease risk allele. PLoS One, 23;8(12):e83958. PMCID: PMC3884107.

3. Dave, M., Papadakis, K.A., Faubion, W.A., Jr. (2014). Immunology of inflammatory bowel disease and molecular targets for biologics. Gastroenterology Clinics of North America., 43(3):405-24. PMID:25110250.

4. Faubion, W.A., Jr, Fletcher, J.G., O'Byrne, S., Feagan, B.G., de Villiers, W.J., Salzberg, B., Plevy, S., Proctor, D.D., Valentine, J.F., Higgins, P.D., Harris, J.M., Diehl, L., Wright, L., Tew, G.W., Luca, D., Basu, K., Keir, M.E. (2014). EMerging BiomARKers in Inflammatory Bowel Disease (EMBARK) study identifies fecal calprotectin, serum MMP9, and serum IL-22 as a novel combination of biomarkers for Crohn's disease activity: role of cross-sectional imaging. American Journal of Gastroenterology,108(12):1891-900. Epub 2013 Oct 15. Erratum in: Am J Gastroenterol. 2014 May;109(5):780. PubMed PMID: 24126633.

5. Cayatte, C., Joyce-Shaikh, B., Vega, F., Boniface, K., Grein, J., Murphy, E., Blumenschein, W.M., Chen, S.,Malinao, M.C., Basham, B., Pierce, R.H., Bowman, E.P., McKenzie, B.S., Elson, C.O., Faubion, W.A., Malefyt Rde, W., Kastelein, R.A., Cua, D., McClanahan, T.K., Beaumont, M. (2012). Biomarkers of Therapeutic Response in the IL-23 Pathway in Inflammatory Bowel Disease. Clinical and translational Gastroenterology, ;3:e10. PubMed Central PMCID: PMC3365674.

6. van Driel, B,, Liao, G., Romero, X., O'Keeffe, M.S., Wang, G., Faubion, W.A., Berger, S.B., Magelky, E.M., Manocha, M., Azcutia, V., Grisham, M., Luscinskas, F.W., Mizoguchi, E., de Waal Malefyt, R., Reinecker, H.C., Bhan, A.K., Wang, N., Trehorst, C. (2012) Signaling lymphocyte activation molecule regulates development of colitis in mice. Gastroenterology, 143(6):1544-1554.e7. PubMed Central: PMCID: PMC3578298.

VERTEBRATE ANIMALS:

The mice used in this proposal will be from protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Mayo Clinic assures strict compliance with all federal regulations and guidelines involving the use of laboratory animals in biomedical research. Animal use assurances have been filed with the IACUC and the NIH. These procedures are closely adhered to and monitored within the institution. All protocols presently being conducted and those proposed, regardless of funding sources, are reviewed and must be approved before their initiation in accordance with these assurance statements. All protocols are active for 3 years after the approval date and adhere to Mayo Clinic and NIH policies on the humane care of animals. All animals will be maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited and centralized facility located in the Guggenheim Building.

1. Provide a detailed description of the use of animals in the research. Identify species, strains, ages, sex, and numbers of animals to be used.

This proposal will use the following mouse lines: 1. FOXP3$^{\Delta EZH2}$ 2. B6129S-Tg(Foxp3-EGFP/cre)1aJbs/J 3. *Foxp3*$^{tm9(EGFP/cre/ERT2)Ayr}$/J 4. FOXP3$^{\Delta IL6R}$ 5. C57/BL6^{CAR} 6. C57BL/6-Foxp3^{tm1Flv}/J (FOXP3 RFP mouse) 7. B6.129S7-*Rag1*^{tm1Mom}/J 8. C57/BL6 EZH2$^{flox/flox}$
These animals are currently being bred in our lab under IACUC# **A31013**. Below we iterate the animal requirements by experimental protocol. Total anticipated animal requirements are listed below each protocol.
1.1, 2.1, and 2.2: Co-IP, PLA, and HMT assay of WT and mutant constructs nucleofected or transduced into WT CD4+ lymphocytes, IL6Rα mutant Treg cells, and FOXP3$^{\Delta EZH2}$ Treg cells.
Each spleen provides 4x10^6 naïve CD4+ T cells and 1x10^5 Treg cells. In review of the recent conduct of similar experiments generating preliminary data, our laboratory utilizes approximately 3-5 WT donor mice per week (approximately 150 per year), 2 IL6Rα mutant mice (approximately 100 per year), and 2 FOXP3$^{\Delta EZH2}$ mice (approximately 100 per year). We have performed experiments on both male and female WT mice with similar results thus plan to use both. Age is 4-6 weeks.
Total WT=150/yr
Total IL6Rα mutant=100/yr
Total FOXP3$^{\Delta EZH2}$=100/yr

1.2: ChIP assay post viral transduction of mutant contructs into FOXP3$^{\Delta EZH2}$ mice. We perform ChIP assay on transduced cells approximately 2x/month, or approximately 25 mice per year. Gender and age as above.
Total FOXP3$^{\Delta EZH2}$=25/yr

1.3, 2.3, 3.1, and 3.2: *In vitro* suppression assay and CD45Rb^{hi} transfer model. The *in vitro* suppression model requires approximately 1x10^5 Treg cells per assay, performed in triplicate (acquired from approximately 3 spleens). One donor WT animal is all that's required to provide the T responder cells. Given at least three mutant EZH2 constructs to test and the requirement to perform the experiment in triplicate to maintain scientific integrity, we anticipate using 27 FOXP3$^{\Delta EZH2}$ mice in protocol 1.3 (3 spleens*3 constructs*triplicate=27) and 9 WT mice (1 spleen*3*triplicate=9).
Total FOXP3$^{\Delta EZH2}$=27

For the *in vivo* colitis experiments, one requires 4 B6.129S7-*Rag1*^{tm1Mom}/J recipients per treatment group. This number of mice was calculated based upon a one-way ANOVA analysis to allow 90% power when the differences between colitis are 1.25 standard deviations more than the controls. Given rescue with at least three constructs to test and the requirement to perform the experiment in triplicate to maintain scientific integrity, we anticipate using 36 RAG^{null} recipients and 10-15 donor mice for a total of approximately <u>50 mice</u> in protocol 1.3. Similarly for the active colitis treatment protocol of 3.2, we will rescue RAG^{null} recipients with IL6R mutant Treg cells or FOXP3$^{\Delta EZH2}$ Treg cells transduced with three EZH2 constructs. Using the same calculations and justification as above, each colitis permutation requires 4 RAG^{null} recipients, repeated 3 times=12 total RAG^{null} animals. As protocol 3.2 tests 4 Treg variants, we will require 48 total RAG^{null} animals. The addition of 10-15 donor mice to the 48 total brings the total anticipated animal requirements to approximately <u>65 animals</u>.
Total RAG^{null} mice: 84 mice

Total IL6R mutant donor mice: 10
Total FOXP3$^{\Delta EZH2}$ donor mice: 30

Neonatal transfer experiments of 3.1: Litters of mouse pairs predicted by genotype to produce FOXP3$^{\Delta EZH2}$ pups will be injected ip with 1x10^{6} WT Treg cells labeled with RFP (C57BL/6-Foxp3^{tm1Flv}/J donor mice). Experience demonstrates 1-2 appropriate FOXP3$^{\Delta EZH2}$ pups per litter; thus we will need to perform this experiment on 3 litters, requiring 12 FOXP3 RFP donor mice.
We will also need to maintain breeding pairs to continuously generate the lines being used in the experiments. The breeding of the FOXP3$^{\Delta EZH2}$ mouse is intense, as CRE+, heterozygous EZH2$^{WT/flox}$ mice need to be bred to the C57/BL6 EZH2$^{flox/flox}$ mouse to produce the appropriate genotype (typically 20% of any litter). This is the primary rationale to the generation of the tamoxifen inducible FOXP3$^{\Delta EZH2}$ mouse line. These adult animals, upon tamoxifen induction of CRE, will serve as donors of EZH2-deficient Treg cells. For every 6 animals used in an experiment, typically 1 breeder pair is required.

Thus total mouse requirements by strain:
WT=150/yr * 5yr=750
WT breeding=125
IL6mt=100/yr*5=500
IL6mt breeding=83
FOXP3$^{\Delta EZH2}$=100/yr*5=500
FOXP3$^{\Delta EZH2}$ breeding=83
RAG^{null}=84
RAG^{null} breeding=14
TOTAL=2139 mice over the 5 year period of the grant (428 mice per year).

All newborn animals with the incorrect genotype will be euthanized before they are 3 weeks old, unless they can be used for breeding. The health of the animals will be monitored on a regular basis. Any animals showing signs of excessive stress, pain, or discomfort during the treatment period will be euthanized as described below. At the end of the treatment cycles, colons will be excised and embedded for histological analysis of inflammation.

2. Justify the use and number of animals and choice of species with additional justification if animals are in short supply or are costly or if you plan to use large numbers.

As principal investigator, I have determined, by means of the following sources, searches, or methods, that alternatives to the procedures which may cause animal pain or distress proposed in this protocol are not available and that this protocol does not unnecessarily duplicate previous experiments. Sources searched include: Medline Current Research Information Services (CRIS) Animal Welfare Information Center (AWIC - National Agriculture Library) PUBMED. The key words that were used in the search (e.g., MESH headings): animal model; colitis; FOXP3; IL6; EZH2. We covered the years 1980 – 2014. Mice are required for the above experiments as there is no safe human options or in vitro options. The complex questions regarding the development of Treg cells in the intestinal milieu cannot be answered *in vitro*. The justification of animal numbers is above with experimental procedures.
There is currently no other experimental system that looks at epigenetic changes resulting in colitis prevention that is comparable to the combination of animals brought together in these studies. Mice are needed because the focus of the project is to determine the role of EZH2 in the development of coitis. These studies cannot be performed without a few key animal models with selective gene deletions. We will limit the number of mice required. To maintain breeding of the various animals, as well as longitudinal studies, we estimate it will be necessary to carry approximately 85 cages of mice on a regular basis (5 adult, same gender mice/cage).

3. Provide information on veterinary care for the animals.

Animals are housed in a pathogen-free laminar flow facility located in the Mayo Clinic Animal Facility on the 19th and 20th floors of the Guggenheim Research Building. The animal facility has controlled temperature, humidity and lighting. Three full-time veterinarians certified in laboratory animal medicine and veterinary pathology will oversee all aspects of animal care in this facility. Beyond the provision of food, water and

housing (that is inspected daily), no specific veterinary care is anticipated. The animal care facilities and use program meets all federal regulations and guidelines. Mayo Foundation is registered with the USDA (41-R-006) as an animal research facility and maintains an NIH animal assurance statement (A3291-01) with the Office of Laboratory Animal Welfare.

4. Describe procedures for ensuring that discomfort, pain, and injury will be limited to what is unavoidable. Describe the use of analgesic, anesthetic, tranquilizing drugs, and restraining devices to minimize discomfort, distress, pain, and injury.

Mayo Clinic strives to ensure that the institutional facilities and procedures adhere in all respects to USDA regulations and NIH guidelines for care and use of laboratory animals. Investigators are required to administer appropriate analgesics to all animals associated with a procedure that would normally require pain medication in humans. The mice are cared for in a clean, climate-controlled environment, with a controlled light-dark cycle. They are fed and watered daily, and cages cleaned weekly. Experiments are ended at pre-defined time points OR when animals have lost more than 20% of the starting body weight or exhibit a moribund appearance. Animals are weighed and examined by the study team daily while in study. Mice will be maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS Guide for the Care and Use of Laboratory Animals.

During adoptive cell transfer, mice are briefly restrained in a clean, warm plexiglass cylinder to allow safe transfer of cells by tail vein injection. Mice are treated with Tylenol overnight following adoptive transfer.

5. Describe any euthanasia method to be used and the reasons for its selection. State whether this method is consistent with the recommendations of the American Veterinary Medical Association Guidelines on Euthanasia. If not, justify not following the recommendations.

Requests to perform euthanasia are reviewed and approved by our IACUC before initiation. Euthanasia may be performed as required by protocol to terminate a study, or as a means to relieve pain or distress that cannot be alleviated by analgesics, or if deemed necessary by the consulting veterinarian. We utilize CO(2) inhalation for euthanasia for the following reasons. (1) The rapid depressant, analgesic, and anesthetic effects of CO2 are well established. (2) Carbon dioxide is readily available and can be purchased in compressed gas cylinders. (3) Carbon dioxide is inexpensive, nonflammable, nonexplosive, and poses minimal hazard to personnel when used with properly designed equipment. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

BIBLIOGRAPHY AND REFERENCES CITED:

1. Rahman, M. K., Midtling, E. H., Svingen, P. A., Xiong, Y., Bell, M. P., Tung, J., Smyrk, T., Egan, L. J., and Faubion, W. A., Jr. (2010) The pathogen recognition receptor NOD2 regulates human FOXP3+ T cell survival. *J Immunol* **184**, 7247-7256
2. Pasare, C., and Medzhitov, R. (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033-1036
3. Xiong, Y., Khanna, S., Grzenda, A. L., Sarmento, O. F., Svingen, P. A., Lomberk, G. A., Urrutia, R. A., and Faubion, W. A., Jr. (2012) Polycomb antagonizes p300/CREB-binding protein-associated factor to silence FOXP3 in a Kruppel-like factor-dependent manner. *J Biol Chem* **287**, 34372-34385
4. Arvey, A., van der Veeken, J., Samstein, R. M., Feng, Y., Stamatoyannopoulos, J. A., and Rudensky, A. Y. (2014) Inflammation-induced repression of chromatin bound by the transcription factor Foxp3 in regulatory T cells. *Nat Immunol* **15**, 580-587
5. Zheng, Y., Josefowicz, S. Z., Kas, A., Chu, T. T., Gavin, M. A., and Rudensky, A. Y. (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**, 936-940
6. DuPage, M., Chopra, G., Quiros, J., Rosenthal, W. L., Morar, M. M., Holohan, D., Zhang, R., Turka, L., Marson, A., and Bluestone, J. A. (2015) The chromatin-modifying enzyme Ezh2 is critical for the maintenance of regulatory T cell identity after activation. *Immunity* **42**, 227-238
7. Yang, X.-P., Jiang, K., Hirahara, K., Vahedi, G., Afzali, B., Sciume, G., Bonelli, M., Sun, H.-W., Jankovic, D., Kanno, Y., Sartorelli, V., O'Shea, J. J., and Laurence, A. (2015) EZH2 is crucial for both differentiation of regulatory T cells and T effector cell expansion. *Sci. Rep.* **5**
8. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing. *Science* **298**, 1039-1043
9. Cha, T. L., Zhou, B. P., Xia, W., Wu, Y., Yang, C. C., Chen, C. T., Ping, B., Otte, A. P., and Hung, M. C. (2005) Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science* **310**, 306-310
10. Chen, B., Liu, J., Chang, Q., Beezhold, K., Lu, Y., and Chen, F. (2013) JNK and STAT3 signaling pathways converge on Akt-mediated phosphorylation of EZH2 in bronchial epithelial cells induced by arsenic. *Cell Cycle* **12**, 112-121
11. Kim, E., Kim, M., Woo, D. H., Shin, Y., Shin, J., Chang, N., Oh, Y. T., Kim, H., Rheey, J., Nakano, I., Lee, C., Joo, K. M., Rich, J. N., Nam, D. H., and Lee, J. (2013) Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell* **23**, 839-852
12. Palacios, D., Mozzetta, C., Consalvi, S., Caretti, G., Saccone, V., Proserpio, V., Marquez, V. E., Valente, S., Mai, A., Forcales, S. V., Sartorelli, V., and Puri, P. L. (2010) TNF/p38alpha/polycomb signaling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration. *Cell Stem Cell* **7**, 455-469
13. Wei, Y., Chen, Y. H., Li, L. Y., Lang, J., Yeh, S. P., Shi, B., Yang, C. C., Yang, J. Y., Lin, C. Y., Lai, C. C., and Hung, M. C. (2011) CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. *Nat Cell Biol* **13**, 87-94
14. Raffin, C., Pignon P Fau - Celse, C., Celse C Fau - Debien, E., Debien E Fau - Valmori, D., Valmori D Fau - Ayyoub, M., Ayyoub, M., Kimura, A., Naka T Fau - Kishimoto, T., Kishimoto, T., Valencia, X., Stephens G Fau - Goldbach-Mansky, R., Goldbach-Mansky R Fau - Wilson, M., Wilson M Fau - Shevach, E. M., Shevach Em Fau - Lipsky, P. E., Lipsky, P. E., Pasare, C., and Medzhitov, R. Human memory Helios- FOXP3+ regulatory T cells (Tregs) encompass induced Tregs that express Aiolos and respond to IL-1beta by downregulating their suppressor functions IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells TNF downmodulates the function of human CD4+CD25hi T-regulatory cells Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells.
15. Kimura, A., Naka, T., and Kishimoto, T. (2007) IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci U S A* **104**, 12099-12104
16. Valencia, X., Stephens, G., Goldbach-Mansky, R., Wilson, M., Shevach, E. M., and Lipsky, P. E. (2006) TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood* **108**, 253-261

17. Voo, K. S., Wang, Y. H., Santori, F. R., Boggiano, C., Wang, Y. H., Arima, K., Bover, L., Hanabuchi, S., Khalili, J., Marinova, E., Zheng, B., Littman, D. R., and Liu, Y. J. (2009) Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* **106**, 4793-4798
18. Dominguez-Villar, M., Baecher-Allan, C. M., and Hafler, D. A. (2011) Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat Med* **17**, 673-675
19. Desreumaux, P., Foussat, A., Allez, M., Beaugerie, L., Hebuterne, X., Bouhnik, Y., Nachury, M., Brun, V., Bastian, H., Belmonte, N., Ticchioni, M., Duchange, A., Morel-Mandrino, P., Neveu, V., Clerget-Chossat, N., Forte, M., and Colombel, J. F. (2012) Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* **143**, 1207-1217
20. Xiong, Y., Svingen, P. A., Sarmento, O. O., Smyrk, T. C., Dave, M., Khanna, S., Lomberk, G. A., Urrutia, R. A., and Faubion, W. A., Jr. (2014) Differential Coupling of KLF10 to Sin3-HDAC and PCAF Regulates the Inducibility of the FOXP3 Gene. *Am J Physiol Regul Integr Comp Physiol* **18**
21. Papadakis, K. A., Krempski, J., Reiter, J., Svingen, P., Xiong, Y., Sarmento, O. F., Huseby, A., Johnson, A. J., Lomberk, G. A., Urrutia, R. A., and Faubion, W. A. (2015) Kruppel-like factor KLF10 regulates transforming growth factor receptor II expression and TGF-beta signaling in CD8+ T lymphocytes. *Am J Physiol Cell Physiol* **308**, 3
22. Sarmento, O. F., Svingen, P. A., Xiong, Y., Xavier, R. J., McGovern, D., Smyrk, T. C., Papadakis, K. A., Urrutia, R. A., and Faubion, W. A. (2015) A novel role for KLF14 in T regulatory cell differentiation. *Cell Mol Gastroenterol Hepatol* **1**, 188-202
23. Mathison, A., Grzenda, A., Lomberk, G., Velez, G., Buttar, N., Tietz, P., Hendrickson, H., Liebl, A., Xiong, Y. Y., Gores, G., Fernandez-Zapico, M., Larusso, N. F., Faubion, W., Shah, V. H., and Urrutia, R. (2013) Role for Kruppel-like transcription factor 11 in mesenchymal cell function and fibrosis. *PLoS One* **8**
24. Okou, D. T., Mondal, K., Faubion, W. A., Kobrynski, L. J., Denson, L. A., Mulle, J. G., Ramachandran, D., Xiong, Y., Svingen, P., Patel, V., Bose, P., Waters, J. P., Prahalad, S., Cutler, D. J., Zwick, M. E., and Kugathasan, S. (2014) Exome sequencing identifies a novel FOXP3 mutation in a 2-generation family with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* **58**, 561-568
25. Rahman, M. K., Offord, C. P., Khanna, S., Ford, G. C., Perrson, X. M., Svingen, P. A., Xiong, Y., Bajzer, Z., and Faubion, W. A., Jr. (2013) Regulatory T cell kinetics in the peripheral blood of patients with Crohn's disease. *Hum Immunol* **74**, 145-150
26. Grzenda, A., Lomberk, G., Svingen, P., Mathison, A., Calvo, E., Iovanna, J., Xiong, Y., Faubion, W., and Urrutia, R. (2013) Functional characterization of EZH2beta reveals the increased complexity of EZH2 isoforms involved in the regulation of mammalian gene expression. *Epigenetics Chromatin* **6**, 1756-8935
27. Mills, L. D., Zhang, L., Marler, R., Svingen, P., Fernandez-Barrena, M. G., Dave, M., Bamlet, W., McWilliams, R. R., Petersen, G. M., Faubion, W., and Fernandez-Zapico, M. E. (2014) Inactivation of the Transcription Factor GLI1 Accelerates Pancreatic Cancer Progression. *J Biol Chem* **289**, 16516-16525
28. Dave, M., Hayashi, Y., Gajdos, G. B., Smyrk, T. C., Svingen, P. A., Kvasha, S. M., Lorincz, A., Dong, H., Faubion, W. A., Jr., and Ordog, T. (2015) Stem cells for murine interstitial cells of cajal suppress cellular immunity and colitis via prostaglandin e2 secretion. *Gastroenterology* **148**, 978-990
29. Cayatte, C., Joyce-Shaikh, B., Vega, F., Boniface, K., Grein, J., Murphy, E., Blumenschein, W. M., Chen, S., Malinao, M. C., Basham, B., Pierce, R. H., Bowman, E. P., McKenzie, B. S., Elson, C. O., Faubion, W. A., Malefyt Rde, W., Kastelein, R. A., Cua, D., McClanahan, T. K., and Beaumont, M. (2012) Biomarkers of Therapeutic Response in the IL-23 Pathway in Inflammatory Bowel Disease. *Clin Transl Gastroenterol*, 2
30. Dave, M., Papadakis, K. A., and Faubion, W. A., Jr. (2014) Immunology of inflammatory bowel disease and molecular targets for biologics. *Gastroenterol Clin North Am* **43**, 405-424
31. Faubion, W. A., Jr., Fletcher, J. G., O'Byrne, S., Feagan, B. G., de Villiers, W. J., Salzberg, B., Plevy, S., Proctor, D. D., Valentine, J. F., Higgins, P. D., Harris, J. M., Diehl, L., Wright, L., Tew, G. W., Luca, D., Basu, K., and Keir, M. E. (2013) EMerging BiomARKers in Inflammatory Bowel Disease (EMBARK) study identifies fecal calprotectin, serum MMP9, and serum IL-22 as a novel combination of biomarkers for Crohn's disease activity: role of cross-sectional imaging. *Am J Gastroenterol* **108**, 1891-1900
32. Kauder, S. E., Santell, L., Mai, E., Wright, L. Y., Luis, E., N'Diaye, E. N., Lutman, J., Ratti, N., Sa, S. M., Maun, H. R., Stefanich, E., Gonzalez, L. C., Graham, R. R., Diehl, L., Faubion, W. A., Jr., Keir, M. E.,

Young, J., Chaudhuri, A., Lazarus, R. A., and Egen, J. G. (2013) Functional consequences of the macrophage stimulating protein 689C inflammatory bowel disease risk allele. *PLoS One* **8**

33. Li, H., Chan, L., Bartuzi, P., Melton, S. D., Weber, A., Ben-Shlomo, S., Varol, C., Raetz, M., Mao, X., Starokadomskyy, P., van Sommeren, S., Mokadem, M., Schneider, H., Weisberg, R., Westra, H. J., Esko, T., Metspalu, A., Kumar, V., Faubion, W. A., Yarovinsky, F., Hofker, M., Wijmenga, C., Kracht, M., Franke, L., Aguirre, V., Weersma, R. K., Gluck, N., van de Sluis, B., and Burstein, E. (2014) Copper metabolism domain-containing 1 represses genes that promote inflammation and protects mice from colitis and colitis-associated cancer. *Gastroenterology* **147**, 184-195
34. van Driel, B., Liao, G., Romero, X., O'Keeffe, M. S., Wang, G., Faubion, W. A., Berger, S. B., Magelky, E. M., Manocha, M., Azcutia, V., Grisham, M., Luscinskas, F. W., Mizoguchi, E., de Waal Malefyt, R., Reinecker, H. C., Bhan, A. K., Wang, N., and Terhorst, C. (2012) Signaling lymphocyte activation molecule regulates development of colitis in mice. *Gastroenterology* **143**, 1544-1554
35. Rudra, D., deRoos, P., Chaudhry, A., Niec, R. E., Arvey, A., Samstein, R. M., Leslie, C., Shaffer, S. A., Goodlett, D. R., and Rudensky, A. Y. (2012) Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol* **13**, 1010-1019
36. Denisenko, O., Shnyreva, M., Suzuki, H., and Bomsztyk, K. (1998) Point mutations in the WD40 domain of Eed block its interaction with Ezh2. *Mol Cell Biol* **18**, 5634-5642
37. Cardoso, C., Timsit, S., Villard, L., Khrestchatisky, M., Font√®s, M., and Colleaux, L. (1998) Specific Interaction between the XNP ATR-X Gene Product and the SET Domain of the Human EZH2 Protein. *Human Molecular Genetics* **7**, 679-684
38. Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* **3**, 995-1000
39. Sadlon, T. J., Wilkinson, B. G., Pederson, S., Brown, C. Y., Bresatz, S., Gargett, T., Melville, E. L., Peng, K., D'Andrea, R. J., Glonek, G. G., Goodall, G. J., Zola, H., Shannon, M. F., and Barry, S. C. (2010) Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. *J Immunol* **185**, 1071-1081
40. Zheng, Y., Josefowicz, S. Z., Kas, A., Chu, T. T., Gavin, M. A., and Rudensky, A. Y. (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T. *Nature* **445**, 936-940
41. Li, H., Bitler, B. G., Vathipadiekal, V., Maradeo, M. E., Slifker, M., Creasy, C. L., Tummino, P. J., Cairns, P., Birrer, M. J., and Zhang, R. (2012) ALDH1A1 is a novel EZH2 target gene in epithelial ovarian cancer identified by genome-wide approaches. *Cancer Prev Res* **5**, 484-491
42. Du, J., Huang, C., Zhou, B., and Ziegler, S. F. (2008) Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. *J Immunol* **180**, 4785-4792
43. Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J. M., Chu, T. T., Corcoran, L., Treuting, P., Klein, U., and Rudensky, A. Y. (2009) Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control. *Nature* **458**, 351-356
44. Izcue, A., Hue, S., Buonocore, S., Arancibia-Carcamo, C. V., Ahern, P. P., Iwakura, Y., Maloy, K. J., and Powrie, F. (2008) Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* **28**, 559-570
45. Moritz, L., Christoph, B., Ivaylo, I. I., Sebastian, H., Stefan, W., Clemens, N., Sandrine, P., Andrew, J. M., David, M. V., George, D. Y., Burkhard, B., Dan, R. L., and Markus, F. N. (2009) RORŒ≥-Expressing Th17 Cells Induce Murine Chronic Intestinal Inflammation via Redundant Effects of IL-17A and IL-17F. *Gastroenterology* **136**, 257-267
46. Kleinschek, M. A., Boniface, K., Sadekova, S., Grein, J., Murphy, E. E., Turner, S. P., Raskin, L., Desai, B., Faubion, W. A., de Waal Malefyt, R., Pierce, R. H., McClanahan, T., and Kastelein, R. A. (2009) Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J. Exp. Med.* **206**, 525-534
47. Yang, X. O., Pappu, B. P., Nurieva, R., Akimzhanov, A., Kang, H. S., Chung, Y., Ma, L., Shah, B., Panopoulos, A. D., Schluns, K. S., Watowich, S. S., Tian, Q., Jetten, A. M., and Dong, C. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* **28**, 29-39
48. Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E., Mak, T. W., Kamradt, T., and Lohoff, M. (2007) The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* **8**, 958-966

49. Huber, M., Brustle, A., Reinhard, K., Guralnik, A., Walter, G., Mahiny, A., von Low, E., and Lohoff, M. (2008) IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci U S A* **105**, 20846-20851
50. Liu, Z., Geboes, K., Colpaert, S., Overbergh, L., Mathieu, C., Heremans, H., de Boer, M., Boon, L., D,ÄôHaens, G., Rutgeerts, P., and Ceuppens, J. L. (2000) Prevention of Experimental Colitis in SCID Mice Reconstituted with CD45RBhigh CD4+ T Cells by Blocking the CD40-CD154 Interactions. *The Journal of Immunology* **164**, 6005-6014
51. Holm, T. L., Poulsen, S. S., Markholst, H., and Reedtz-Runge, S. (2012) Pharmacological Evaluation of the SCID T Cell Transfer Model of Colitis: As a Model of Crohn's Disease. *International Journal of Inflammation* **2012**
52. Abadia-Molina, A. C., Ji, H., Faubion, W. A., Julien, A., Latchman, Y., Yagita, H., Sharpe, A., Bhan, A. K., and Terhorst, C. (2006) CD48 controls T-cell and antigen-presenting cell functions in experimental colitis. *Gastroenterology* **130**, 424-434
53. Abadia-Molina, A. C., Mizoguchi, A., Faubion, W. A., De Jong, Y. P., Rietdijk, S. T., Comiskey, M., Clarke, K., Bhan, A. K., and Terhorst, C. (2005) In vivo generation of oligoclonal colitic CD4+ T-cell lines expressing a distinct T-cell receptor Vbeta. *Gastroenterology* **128**, 1268-1277
54. de Jong, Y. P., Abadia-Molina, A. C., Satoskar, A. R., Clarke, K., Rietdijk, S. T., Faubion, W. A., Mizoguchi, E., Metz, C. N., Alsahli, M., ten Hove, T., Keates, A. C., Lubetsky, J. B., Farrell, R. J., Michetti, P., van Deventer, S. J., Lolis, E., David, J. R., Bhan, A. K., and Terhorst, C. (2001) Development of chronic colitis is dependent on the cytokine MIF. *Nat Immunol* **2**, 1061-1066
55. Faubion, W. A., De Jong, Y. P., Molina, A. A., Ji, H., Clarke, K., Wang, B., Mizoguchi, E., Simpson, S. J., Bhan, A. K., and Terhorst, C. (2004) Colitis is associated with thymic destruction attenuating CD4+25+ regulatory T cells in the periphery. *Gastroenterology* **126**, 1759-1770
56. Guil, S., Soler, M., Portela, A., Carrere, J., Fonalleras, E., Gomez, A., Villanueva, A., and Esteller, M. (2012) Intronic RNAs mediate EZH2 regulation of epigenetic targets. *Nat Struct Mol Biol* **19**, 664-670
57. Dominitzki, S., Fantini, M. C., Neufert, C., Nikolaev, A., Galle, P. R., Scheller, J., Monteleone, G., Rose-John, S., Neurath, M. F., and Becker, C. (2007) Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. *J Immunol* **179**, 2041-2045
58. Chung, Y., Chang, S. H., Martinez, G. J., Yang, X. O., Nurieva, R., Kang, H. S., Ma, L., Watowich, S. S., Jetten, A. M., Tian, Q., and Dong, C. (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* **30**, 576-587
59. Li, L., Kim, J., and Boussiotis, V. A. (2010) IL-1beta-mediated signals preferentially drive conversion of regulatory T cells but not conventional T cells into IL-17-producing cells. *J Immunol* **185**, 4148-4153
60. Nie, H., Zheng, Y., Li, R., Guo, T. B., He, D., Fang, L., Liu, X., Xiao, L., Chen, X., Wan, B., Chin, Y. E., and Zhang, J. Z. (2013) Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. *Nat Med* **19**, 322-328
61. Nadkarni, S., Mauri, C., and Ehrenstein, M. R. (2007) Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *J Exp Med* **204**, 33-39
62. Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., and Kronenberg, M. (2009) Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* **10**, 1178-1184
63. Komatsu, N., Okamoto, K., Sawa, S., Nakashima, T., Oh-hora, M., Kodama, T., Tanaka, S., Bluestone, J. A., and Takayanagi, H. (2014) Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med* **20**, 62-68
64. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**, 1351-1362
65. Dou, Y., Yao, B., and Zhang, C. (2014) PhosphoSVM: prediction of phosphorylation sites by integrating various protein sequence attributes with a support vector machine. *Amino Acids* **46**, 1459-1469
66. Xue, Y., Ren, J., Gao, X., Jin, C., Wen, L., and Yao, X. (2008) GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol Cell Proteomics* **7**, 1598-1608
67. Sahasrabuddhe, A. A., Chen, X., Chung, F., Velusamy, T., Lim, M. S., and Elenitoba-Johnson, K. S. (2015) Oncogenic Y641 mutations in EZH2 prevent Jak2/beta-TrCP-mediated degradation. *Oncogene* **34**, 445-454
68. McDoom, I., Ma, X., Kirabo, A., Lee, K.-Y., Ostrov, D. A., and Sayeski, P. P. (2008) Identification of Tyrosine 972 as a Novel Site of Jak2 Tyrosine Kinase Phosphorylation and its Role in Jak2 Activation. *Biochemistry* **47**, 8326-8334

69. James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., and Vainchenker, W. (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* **434**, 1144-1148
70. Essletzbichler, P., Konopka, T., Santoro, F., Chen, D., Gapp, B. V., Kralovics, R., Brummelkamp, T. R., Nijman, S. M., and Burckstummer, T. (2014) Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res* **24**, 2059-2065
71. Buttar, N. S., DeMars, C. J., Lomberk, G., Rizvi, S., Bonilla-Velez, J., Achra, S., Rashtak, S., Wang, K. K., Fernandez-Zapico, M. E., and Urrutia, R. (2010) Distinct Role of Kruppel-like Factor 11 in the Regulation of Prostaglandin E2 Biosynthesis. *Journal of Biological Chemistry* **285**, 11433-11444
72. Smigiel, K. S., Srivastava, S., Stolley, J. M., and Campbell, D. J. (2014) Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. *Immunol Rev* **259**, 40-59
73. Lomberk, G., Mathison, A. J., Grzenda, A., Seo, S., DeMars, C. J., Rizvi, S., Bonilla-Velez, J., Calvo, E., Fernandez-Zapico, M. E., Iovanna, J., Buttar, N. S., and Urrutia, R. (2012) Sequence-specific recruitment of heterochromatin protein 1 via interaction with Kruppel-like factor 11, a human transcription factor involved in tumor suppression and metabolic diseases. *J Biol Chem* **287**, 13026-13039
74. Endharti, A. T., Okuno, Y., Shi, Z., Misawa, N., Toyokuni, S., Ito, M., Isobe, K., and Suzuki, H. (2011) CD8+CD122+ regulatory T cells (Tregs) and CD4+ Tregs cooperatively prevent and cure CD4+ cell-induced colitis. *J Immunol* **186**, 41-52
75. Liu, H., Hu, B., Xu, D., and Liew, F. Y. (2003) CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4. *J Immunol* **171**, 5012-5017
76. Uhlig, H. H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J. D., Ramsdell, F., and Powrie, F. (2006) Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during. *J Immunol* **177**, 5852-5860
77. Loddenkemper, C., Maul, J., Berg, E., Stein, H., Zeitz, M., and Duchmann, R. (2006) Analysis of FOXP3 protein expression in human CD4(+)CD25(+) regulatory T cells at. *Eur J Immunol* **36**, 246
78. Gout, T., Ostor, A. J., and Nisar, M. K. (2011) Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: a systematic literature review. *Clin Rheumatol* **30**, 1471-1474
79. Iwasa, T., Nakamura, K., Ogino, H., Itaba, S., Akiho, H., Okamoto, R., Iboshi, Y., Aso, A., Murao, H., Kanayama, K., Ito, T., and Takayanagi, R. (2011) Multiple ulcers in the small and large intestines occurred during tocilizumab therapy for rheumatoid arthritis. *Endoscopy* **43**, 70-72
80. Smolen, J. S., Beaulieu, A., Rubbert-Roth, A., Ramos-Remus, C., Rovensky, J., Alecock, E., Woodworth, T., and Alten, R. (2008) Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. *Lancet* **371**, 987-997
81. Helbling, R., Nyddeger, A., Angelini, F., Von Scheven Gete, A., and Hofer, M. (2013) P03-024 - Early onset IBD treated by tocilizumab. *Pediatric Rheumatology* **11**, A222
82. Lee, E. B., Fleischmann, R., Hall, S., Wilkinson, B., Bradley, J. D., Gruben, D., Koncz, T., Krishnaswami, S., Wallenstein, G. V., Zang, C., Zwillich, S. H., and van Vollenhoven, R. F. (2014) Tofacitinib versus methotrexate in rheumatoid arthritis. *N Engl J Med* **370**, 2377-2386
83. Sandborn, W. J., Ghosh, S., Panes, J., Vranic, I., Su, C., Rousell, S., and Niezychowski, W. (2012) Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med* **367**, 616-624
84. Sarmento, O. F., Svingen, P. A., Xiong, Y., Xavier, R. J., McGovern, D., Smyrk, T. C., Papadakis, K. A., Urrutia, R. A., and Faubion, W. A. A novel role for KLF14 in T regulatory cell differentiation.
85. Ma, A. C., Lee, H. B., Clark, K. J., and Ekker, S. C. (2013) High efficiency In Vivo genome engineering with a simplified 15-RVD GoldyTALEN design. *PLoS One* **8**
86. Wu, X., Blackburn, P. R., Tschumper, R. C., Ekker, S. C., and Jelinek, D. F. (2014) TALEN-mediated genetic tailoring as a tool to analyze the function of acquired mutations in multiple myeloma cells. *Blood Cancer J* **9**, 32
87. Cao, T., Wenzel, S. E., Faubion, W. A., Harriman, G., and Li, L. (2010) Enhanced suppressive function of regulatory T cells from patients with immune-mediated diseases following successful ex vivo expansion. *Clinical Immunology* **136**, 329-337

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200 First St SW
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[redacted]

William Faubion, M.D.
Mayo Clinic
[redacted]

June 18, 2015

Dear Bill,

I am happy to provide this letter of collaboration in support of your R01 proposal entitled "**Inflammatory cascades disrupt Treg function through epigenetic mechanisms.**" As the director of the Genetics and Model Systems Core, my passion has been to develop genetic editing tools as a service line for our investigators engaged in the NIH-funded Center for Cell Signaling, Mayo Clinic. As we discussed, we have deep experience in several tools that will be of use in your system including CRISPRs, TALENs, and the Sleeping Beauty and Tol2 transposon systems. Your choice to utilize TALENs to edit primary T cells makes sense given our published experience in the genetic editing of lymphoid cells lines with this system. We are also ready to help you try other tools such as the rapidly developing CRISPR/Cas9 systems as they become available. I look forward to our collaboration, particularly as it relates to the genetic editing of WT lymphocytes and the treatment of active colitis with Treg cellular therapy.

I wish you every success with your application and look forward to continuing collaboration on this interesting project.

Sincerely,
[redacted]

Stephen C. Ekker, Ph.D.
Professor of Biochemistry and Molecular Biology
Director, Mayo Addiction Research Center
Editor-in-chief, the *Zebrafish* journal

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June 18, 2015

William A. Faubion, M.D.
Division of Gastroenterology and Hepatology
Assistant Professor of Medicine
Mayo Clinic
200 First Street, SW
Rochester, MN 55905

Dear Bill,

I am happy to provide this letter of collaboration in support of your R01 proposal entitled "**Inflammatory cascades disrupt Treg function through epigenetic mechanisms.**" As we recently discussed in person, I am looking forward to our collaboration on the role of IL6 signaling and Treg function. As you know, we have developed an animal line with a spontaneous point mutation in the IL6 receptor leading to an absence in functional protein. I think this mouse line will be of interest to you, as you develop your assays of EZH2 post-translational modification downstream of IL6, and the treatment protocols of active colitis. We have been happy to provide fresh cells for your studies and look forward to the development of a breeding colony in your laboratory. Congratulations on your proposal and I look forward to our continued collaboration.

Sincerely,

[redacted]

Chandrashekhar Pasare, Ph.D.

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Mayo Clinic
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Thomas C. Smyrk, M. D.
Division of Anatomic Pathology
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June 18, 2015

William A. Faubion, M. D.
Gastroenterology and Hepatology
Mayo 9 East

Dear Bill:

I am happy to provide this letter of collaboration in support of your ROl proposal entitled **"Inflammatory cascades disrupt Treg function through epigenetic mechanisms."** In my role as an expert GI pathologist, I look forward to our continued collaboration as it relates to interpretation of colitis in your murine models. We have used our blinded histologic scoring assay successfully in our recent collaborative papers. I look forward to our continued work in your new studies ofthe treatment of active colitis with Treg cellular therapy. Congratulations on your proposal and I look forward to our continued work together,

Sincerely,
[redacted]

Thomas C. Smyrk, M.D.

TCS/cjh

MAYO CLINIC
Rochester, Minnesota

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Professor of Biophysics and Medicine
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Mayo Clinic College of Medicine
Rochester, MN 55905
[redacted]

June 18, 2015

Dear Bill,

As you know, my habit is not to write support letters for NIH proposal unless I really believe that they have the potential to reach the outstanding category.

For this reason, it is with great enthusiasm that I put my pen to paper to express my excitement about this proposal. I have read it carefully and I believe that you focus on a remarkably exciting are of research, which is currently underrepresented though of great biomedical significance. This proposal logically addresses critical mechanistic questions of high implications, and the data provided indicate that it is highly feasible. Therefore, I congratulate you for your efforts.

Noteworthy, however, I would also like to complement this letter by expressing that it has been a pleasure for our laboratory to collaborate with you on the epigenetics, a subject on which my laboratory has made significant discoveries. We have also enjoyed our discussions and your presentations and open sharing of the data. Therefore, I assure you that you can count with all the conceptual framework, expertise, reagents, personnel and equipment from my laboratory to successfully help to achieve the goals outlined by you in the grant. Specifically, our work on EZH2 is in line with your proposed investigations. As our previous collaborative work suggests, I think your investigation into the regulation of this 3MeK27 writer HMT and immune cell function is mechanistically insightful and biomedically significant. As always, you have full access to our extensive collection of constructs, inhibitors, animal models, and other reagents you will find useful in your work. In particular, our previous work on AKT, STAT3, and EZH2 led to a large inventory of reagents (inhibitors, gain of function mutants, etc) relevant to your work. Additionally, I am happy to provide expertise in the use of our protein chromatography system as you look into the EZH2, FOXP3 protein complex.

Lastly, the most important aspect of your proposal, I believe is that it will generate fundamental data, which can be applied to other systems in addition to your area of current focus. Therefore, this is what I call, a “proposal with a significant added value” which can benefit more areas than the one studied in the grant.

For all these reasons, I am looking forward to our collaborations, discussions, and planning of additional experiments. In simple words, my commitment to this proposal is the strongest that I can express.

Good Luck with your thoughtful and exciting experiments and I look forward to continuing with our already ongoing fruitful interactions.

Best Regards,

[redacted]

Raul Urrutia, MD
Professor of Biophysics and Medicine
Director, Epigenetics and Chromatin Dynamics Laboratory
Director, Epigenomics Education, Mayo Clinic

RESOURCE SHARING PLAN:

Sharing of Data and Model Organisms with the Broader Research Community:
-We will comply with all NIH Public Access Policies including depositing peer-reviewed publications resulting from this research into National Library of Medicine PubMed Central within 12 months after the official date of publication.

-Additionally, all cell and mouse lines that are generated will be made available as requested by other investigators pursuant to Mayo Clinic and NIH guidelines.