Modulation of HIV-1 replication and T cell activation by FoxP3

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Abstract

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FoxP3 modulation of HIV-1 Replication and T cell activation

(Under the direction of Dr. Lishan Su)

Regulatory T cells (Treg) are defined as the population of CD4+CD25+ that express

the forkhead transcription factor family member FoxP3. Treg cells are characterized by the

inability to produce IL-2 upon stimulation, defects in *in vitro* proliferation, and the ability to

suppress effector T cell (Teff) activation. FoxP3 plays a crucial role in Treg function and is

thought to be a factor required for the development and maintenance of Tregs. We first set

out to describe the role of FoxP3 in the regulation of HIV LTR activation in Treg cells. Upon

primary Treg infection with HIV-1 NL4-luciferase reporter virus, we observed increased

transcription of the LTR. Similarly, primary T cells or Jurkat T cells transduced with

retrovirus expressing Foxp3 showed a similar enhancement of HIV-1 LTR. We demonstrate

that Foxp3 enhancement of LTR requires intact NF-κB binding sites, and FoxP3 expression is

associated with increased binding of NF-κB at the core enhancer of HIV-1 LTR and

differential histone acetylation of the LTR and *IL-2* promoters.

We demonstrate that FoxP3 expression in Jurkat T cells and primary Tregs inhibits

global HDAC activity. Specifically, Foxp3 inhibits HDAC1 activity and this function requires

the proline-rich domain. We show that FoxP3 is present in a large molecular weight complex with HDAC1, and interacts with HDAC1 in T cells. A point mutation in the forkhead domain that inhibits Foxp3 function in Tregs *in vivo* is unable to associate with and inhibit HDAC1 activity, suggesting the importance of FoxP3/HDAC1 interaction for HDAC1 inhibition. Finally, we demonstrate knockdown of HDAC1 inhibits FoxP3 regulation of *IL-2* expression and HIV-1 LTR activity in T cells.

Lastly, we report that FoxP3 interacts with the ATPase nucleosome remodeler Mi-2β in T cells. This interaction requires the zinc-finger domain, and FoxP3 expression is associated with a decreased binding of Mi-2β at the *IL-2* promoter. We also demonstrate that knockdown of Mi-2β inhibits *IL-2* expression, supporting a model for FoxP3-mediated repression of transcription through the removal of factors required for optimal gene expression. Thus, FoxP3 regulates gene expression at multiple levels, from transcription factor modulation to chromatin remodeling factor regulation.

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List of Abbreviations

AIDS   Acquired Immune-deficiency Syndrome

APC   antigen presenting cells

ART   anti-retroviral therapy

CMV   Cytomegalovirus

ChIP   chromatin Immunoprecipitation

Chip   microarray

DAG   diacylglycerol

DBD   DNA-binding domain

DNase   DNA nuclease

ER   Endoplasmic reticulum

Foxp3   Forkhead box P3

Foxp3-C   Foxp3 without N-terminus

Foxp3-LZFKH   Foxp3 with Leucine zipper and forkhead

Foxp3-FKH   Foxp3 with forkhead

GADS   Grb2-related adapter downstream of Shc

GFP   green fluorescent protein

GVHD   graft-versus-host disease

HAT   histone acetyl-transferase

HCV   Hepatitis C virus

HDAC   histone deacetylase

HDACi   histone deacetylase inhibitor

HIV   Human Immunodeficiency virus

HSPG	retrovirus vector
HSV	Herpes Simplex Virus
H3	histone 3
H4	histone 4
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IL-4	Interleukin-4
Iono	Ionomycin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IP3	Inositol-1,4,5-triphosphate
ISWI	imitation SWI
ITAM	immunoreceptor tyrosine based activation motifs
LAT	linker for activation of T cells
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MHC	major histocompatibility complex
MNase	micrococcal nuclease
NaBut	sodium butyrate
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NK	Natural Killer
NuRD	nucleosome remodeling and deacetylation
PIP2	phosphatidylinositol-4,5-bisphosphate

PLCγ1	phospholipase Cγ1
PMA	phorbol 12-myristate 13-acetate
SAHA	suberoylanalide hydroxamic acid
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	Simian Immunodeficiency virus
SOS	son of sevenless
SWI/SNF	switching defecting/sucrose non-fermenting
TCR	T cell Receptor
Teff	effector T cell
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TPX	trapoxin
Treg	regulatory T cell
TSA	trichostatin-A
VPA	valproic acid
VSV-g	vesicular stomatitis virus glycoprotein

Chapter I

Introduction

Introduction

Introduction Forward: The primary goal of my graduate tenure and this introduction is to be as comprehensive and concise as possible, leaving no stone unturned unless technical difficulties inhibit my progress. Therefore, I will not be giving a complete review of the HIV lifecycle for fear of boredom and for the fact that it has been extensively reviewed in the literature. Therefore I will refer you to a fantastic review by Eric Freed (1) for a comprehensive look at HIV-1 replication. Thank you and enjoy the following introduction to HIV, Foxp3 and Regulatory T cells.

Part I: HIV infection overview

Brief History and Emergence of HIV

The earliest documented case of HIV-1 infection came from a stored serum sample collected in 1959 from an adult Bantu male in the Democratic Republic of Congo (2). Similarly, HIV-2 ancestors have dated back to the 1940. Estimates by Korber *et al.* put the timing of HIV-1 M group divergence somewhere in the 1930's (3). Currently, it is proposed that HIV-1 came to humans from the chimpanzee, while HIV-2 has origins from the sooty mangabey, which harbor SIVcpz and SIVsm respectively (4-6). In SIVsm's natural host, which includes chimpanzees, sooty mangabeys and African green monkeys, SIVsm does not cause overt disease despite high viral load. It is not until it is

present in an "unnatural" host (Rhesus macaque and humans for SIVsm and HIV, respectively) that disease progression is associated with chronic immune activation, T cell depletion and subsequent immunodeficiency. This intriguing difference has formed the foundation for studies to determine the mechanisms of immunopathogenesis in HIV infection.

Acute infection: A shift in the paradigm

HIV disease progression can be separated into 3 distinct phases: (i) acute infection occurring in the first 3-6 weeks in humans and 1-4 weeks in macaques, which is associated with a spike in viral load and a subsequent decrease in viral load to the viral set point; (ii) the chronic phase of infection lasting 6-10 years. This asymptomatic phase coincides with a gradual increase in viral load and decrease in CD4+ T cell counts over time; (iii) and the final phase lasting roughly 12-18 months and is associated with AIDS and immune system failure (7). The focus of past research has been to determine the mechanism of immune activation during the chronic phase and the resultant AIDS progression. More recently, we have shifted our focus on what is happening during the acute phase of infection as a predictor of disease progression. Now, HIV pathogenesis can be divided into two major phases; the acute infection phase associated with a dramatic loss of CD4+ T cells residing in mucosal tissue, and a chronic phase characterized by immune activation and gradual loss of peripheral CD4+ T cells over time (8).

Looking more closely at the acute phase of infection, plasma viral load increase coincides with CD8+ T cell increases and a drop in CD4+ T cell counts (9). This leads to an inversion of the CD4+/CD8+ ratio. Until recently, the magnitude of CD4+ T cell depletion and its consequence were not fully appreciated. Initial studies in 1998 using an SIV model described a profound depletion of CD4+ T cells in both the gut and gut-associated lymphoid tissue (GALT) (10, 11), and more recent studies have described a similar depletion in the gut of HIV-1 infected individuals (12-14). The importance of these findings is underscored by the fact that between 60-80% of the total CD4+ T cell population resides in the gut and associated lymphoid tissue (15). The CD4+ T cell population that is most affected and depleted by SIV and HIV has a resting memory CD4+ phenotype, Ki-67- and CD69-, and CD45RA- (16). Of greater importance is that the majority of mucosal CD4+ T cells are CD45RA-, and up to 75% express the HIV-1 coreceptor required for T cell infection, CCR5 (12, 17, 18). Thus, during the acute stage of infection, the resting memory T cell is a major target of SIV and HIV infection and depletion.

The chronic phase of infection

As previously stated, the chronic phase of HIV is associated with a steady decline in CD4+ T cell numbers, hyper-immune activation, and a slow and steady rise in viral load in patients not on ART. In the gut, the depletion of CD4+ memory T cells continues and the restoration of this population is never achieved in both pathogenic SIV and HIV

infection (11, 12). As a consequence, chronic immune activation occurs due in part to

homeostatic proliferation of the T cell pool to replenish the HIV-depleted pool, and also

microbial translocation leading to LPS-mediated immune activation (19, 20).

Interestingly, not only does HIV affect the dynamics of T cell homeostasis, but the

function of CD4+ T cells is also impaired. HIV-1 specific T cells are preferentially infected

by HIV harbored in dendritic cells (21, 22) and have been shown to consequently lack

effector function. In general, disruption of immune homeostasis and subsequent overt

immune activation are hallmarks of this phase of HIV-1 infection, and defining and

characterizing the mechanisms contributing to immune activation is of great

importance.

Part II: The Role of Regulatory T cells in immune-balance- Do Tregs hold the key?

In order to delineate the complicated mechanism behind HIV pathogenesis and

progression to AIDS, you must first understand mechanisms of immune control.

Therefore, the following section will be dedicated to describing and understanding a key

player in immune homeostasis, the Regulatory T cell (Treg).

Regulatory T cell in the Immune system: A brief history

The regulation of immune tolerance is a critical aspect of immunology. The

balance between recognition of self versus non-self is essential for maintenance of

immune homeostasis. Excessive immune responses can have deleterious effects, as

seen in autoimmune disease or immune pathology from infection or allergic insult.

Similarly, understanding the mechanism of immune tolerance is beneficial in controlling

tissue rejection following transplantation, or reversing unresponsiveness to autologous

tumor cells. The idea of an intrinsic T cell population capable of suppressing the

immune system came to light in 1970 wherein the ability of T cells to both enhance and

suppress immune responses was first described (23). For several years following the

initial discovery, both CD8+ and CD4+ suppressor T cell populations were described, but

the inability to find a biological factor responsible for suppressor T cells led to a decline

in suppressor T cell interest. During the late 1980's, T cell tolerance was discovered to

be associated with mechanisms of clonal T cell deletion in the thymus (24, 25), and the

discovery of secreted suppressive cytokines such as IL-10 further led to our thinking that

a specific T cell subset was not required for tolerance (26).

Simultaneously, work on natural self-tolerance mechanisms was in place with

the discovery in 1969 that removal of the thymus in neonatal mice resulted in ovarian

destruction due to autoimmunity (27). Importantly, removal of the thymus 3 days

following birth resulted in several autoimmune manifestations (28), and work by several

groups demonstrated that CD4+ T cells can be adoptively transferred into T cell-

deficient mice and cause disease (29). Further work by Sakaguchi *et al.* in 1995

demonstrated that a population of CD4+ CD25hi T cells was required for tolerance,

wherein depletion of this population led to overt autoimmune disease while subsequent

addition of CD4+CD25+ T cells could control autoimmunity (30, 31). Thus, there was a

specific T cell population required to control the balance of the immune system.

FOXP3 gene in Tregs

In 2001, the discovery of the role of a transcription factor mediating

autoimmune disease in mice can be considered the most important finding in Treg

biology. A member of the forkhead winged-helix family, Foxp3, when disrupted in the

scurfy mouse resulted in a fatal lymphoproliferative disorder (32). A disorder found in

humans, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked

syndrome), was associated with mutation in the *FOXP3* gene, and was characterized by

autoimmune disease in multiple organs. In 2003, multiple groups were able to show

that Foxp3 protein localized to CD4+CD25+ T cells, *Foxp3* expression was required for

Treg development and function, and expression of Foxp3 by retroviral transduction in

CD4+CD25- T cells conferred a suppressive phenotype (33-35). Through this work it

became evident that Foxp3 is a critical factor in Treg function. A more comprehensive

look into the role of Foxp3 in Treg cells will be discussed in later sections.

Mechanisms of Treg Suppression

Following the discovery of CD4+CD25hi Treg subset, several groups were able to

isolation and further characterize the mechanism of Treg suppression activity. Several

groups showed that Tregs cultured *in vitro* with CD4+CD25- responder cells (or CD8+ T

cells for that matter) along with either APC-mediated stimulation or TCR stimulation with CD3 antibody was able to repress proliferation (36, 37). Not only was it demonstrated that Tregs were able to suppress T cells *in vitro*, but Tregs also were characterized by an inability to produce IL-2, were hypoproliferative *in vitro* upon TCR stimulation, and furthermore, the mechanism of Treg suppression was cell contact-dependent. This lead to the current and ongoing research to discover the molecular mechanisms involved in Treg suppression.

As previously described, ectopic expression of Foxp3 in CD4+ CD25- is able to recapitulate the suppressive phenotype of Tregs almost completely. In terms of Treg development in the thymus, *Foxp3* expression is turned on 3 days following birth, correlating well with the previously described neonatal day 3 thymectomy-induced autoimmune disease (38). Characteristic of Foxp3 expression was the increase in activation marker CD25, and other cell surface markers associated with T cell suppression such as CTLA-4 and GITR (33). Several studies have shown the ability of Tregs to suppress several cell populations and cellular functions, including B cell proliferation and immunoglobulin production, the function of mature dendritic cells, NK and NKT cells, and importantly both naïve and memory CD4+ and CD8+ T cell populations (39-44). Also of importance to note is the ability of Treg cells to suppress in an antigen specific and non-specific manner, the latter resulting in bystander suppression.

To date, several mechanisms of suppression by Tregs have been demonstrated. With respect to cell-to-cell contact, upregulated cell surface factors such as CTLA-4 and LAG-3 have been shown to play a role in Treg suppression *in vivo* as demonstrated by administration of blocking antibodies for the specific factors in mice (45, 46). Others have identified a role for cytokines in the suppressive activity of Tregs. IL-10 secreted locally can control colitis and experimental allergic enchaphalomyelitis (EAE) (47, 48), while TGF-β is expressed on the surface of both human and murine Tregs and is required for suppression (40, 49). An exact mechanism of suppression still remains to be resolved, although it is becoming clear that multiple mechanisms of suppression occur that are heavily dependent on the microenvironment, location of action and cell populations involved.

The Role of Tregs in infection

The concept of the study of regulatory T cells was rejuvenated through work on autoreactive T cells and immune tolerance, but the impact of Tregs doesn't stop there. It was clear that Treg cells would play a major role in infection, and Belkaid *et al.* provided the first evidence of the role of CD4+CD25+ T cells in the control of disease. In this work, *Leishmania major* infection in mice when depleted of Treg cells was unable to establish a persistent infection and was cleared, due in part to a decrease in Treg cells and thus a consequent increase in CD8+ T cell-mediated parasite clearance. This also firmly established a connection between sterilizing immunity and immune memory to

pathogens, and put Tregs in the forefront (50). Within a year of this finding, Treg cells

were also linked to control of the immune response to viral infection (44). In the

following sections, the role of Tregs in viral clearance and immunity will be discussed,

and more significantly, their role in HIV infection.

Since Tregs were now implicated in immune tolerance and pathogen clearance,

several groups set out to describe the role of Tregs in pathogen persistence.

Experiments with Herpes Simplex virus (HSV) infection in mice showed that depletion of

CD4+CD25+ T cells 3 days prior to infection resulted in elevated HSV-specific CD8+ T cell

response *in vivo* in the acute and memory phases, and elevated HSV-specific CD4+ T cell

responses (44, 51). It became clear that the immune response to HSV infection was

increased by Treg removal, wherein better memory response was decreased in the

presence of Tregs, and protective immunity was compromised. In the case of a second

chronic disease, Hepatitis C virus (HCV), the study of Tregs is difficult due to a lack of an

animal model. Regardless, studies done in humans comparing chronic HCV-infected

individuals to HCV-infected and recovered persons demonstrated a significant

difference in the frequency of CD4+CD25+ T cells. Persistently HCV-infected patients

had an increased percentage of Tregs, and the Tregs were able to suppress HCV-specific

CD8+ T cell responses *ex vivo* (52).

The Role of Tregs in HIV infection

Since the role of Tregs in establishing chronic versus acute diseases has been established for several pathogens, the importance of this cell population in HIV-1 infection is of great importance. Since the hallmark of progression to AIDS is the decrease in CD4+ T cells and hyper-immune activation, it is not unreasonable to rationalize the importance of Tregs in chronic immune activation. The question remains if depletion of Tregs in HIV-1-infected patients contributes to uncontrolled immune activation. Thus determining the kinetics and functional response of Tregs is of great importance. Characterization of the role of Treg cells in HIV-1 infection has been controversial, most notably for the lack of consistent determination of a Treg phenotype and technical methods for determining Treg numbers in HIV patients, and lack of understanding of the dynamics of Tregs in peripheral blood over the time course of disease. Thus, several groups have shown that Tregs numbers are either decreased (53-57) or increased (58-61) in HIV-1 infection. A clearer understanding of Tregs in disease progression has come to light due to the use of a monkey model of HIV infection. As previously stated, SIV infection in African green monkeys and sooty mangabeys does not result in AIDS-like disease, while several groups have shown that rhesus macaque infection is an accelerated and consistent model for HIV disease progression (62-65). Thus, using this model, Periera *et al.* demonstrated that Treg numbers in peripheral blood of SIV-infected sooty mangabeys did not change over the course of infection, while there was a severe depletion in rhesus macaques. Interestingly, the function of Tregs *ex vivo* was different between these two monkey models, and the apparent

numerical or function loss of Tregs correlated with viral load (66). Similarly, Chase *et al*. described severe depletion of Treg cells in the gut of SIV-infected rhesus macaques during the acute and chronic phase of the infection, consistent with the finding that the majority of the CD4+CCR5+ T cell population is depleted in acute SIV infection (67). Surprisingly, none of these studies determined the relative level of infection of Tregs compared to memory T cells, although the Treg population was described in brief to harbor genomic SIV.

Given the importance for Tregs in immune-homeostasis, depletion would likely have a dramatic effect on T cell responses. Depletion of T cells by direct infection or bystander apoptosis is well documented in HIV infection, and similarly Tregs in the lymphoid tissue have been shown to be infected by SIV, with approximately 13% of Treg cells being productively infected (58). To date there have been no studies showing direct infection of Tregs in HIV infected individuals, although Tregs express the HIV receptor CD4 and coreceptor CCR5 and are highly susceptible to infection *in vitro* (53). In fact, our group has shown that HIV replicates more efficiently in Tregs than Teff cells (68), although others have demonstrated the opposite effect (69). More recently, our group has shown that in humanized *Rag2$^{-/-}$γc$^{-/-}$* double knockout mice, HIV-1 is able to efficiently infect and deplete Treg cells (Jiang *et al.* manuscript submitted). In this study, they also determined that depletion of Tregs through ONTAK treatment diminished the viral load and impaired T cell infection. Clearly, the role of Treg infection and depletion requires further study to fully delineate their role in immune pathogenesis, viral pathogenesis and control of viral replication in all stages of disease.

PartIII: T cell activation

T cells in the immune system

In vertebrates, the immune system is broken down into two separate divisions to ward off external insults and invading pathogens. The innate immune response is an ancient component of the immune repertoire found in both plants and animals, and is vital in the recognition of infectious nonself from noninfectious self. This arm of immunity is comprised of multiple cell types, including phagocytes such as dendritic cells, macrophages and neutrophils, along with eosinophils, mast cells, basophils and natural killer (NK) cells (70). What distinguishes these cells from the adaptive immune arm is the recognition of nonself antigens with invariant receptors that do not require DNA rearrangement events. Toll-like receptors are germline encoded and evolved to recognize patterns produced by metabolic processes of microbial pathogens but not host products. Conversely, the adaptive immune response uses an entirely different receptor to mount an immune response to a specific pathogen. These T cell receptors (TCR) are produced through somatic mutation and gene segment rearrangement to give a staggering array ($\sim10^8$) of TCR specificity (71). This seemingly random recognition of antigens provides us with a response capable of specificity to foreign antigens and memory of infection, allowing for a faster and more efficient immune response. However, an innate drawback of this type of system is the inability to differentiate self from non-self, as seen in detrimental responses such as autoimmunity, allergy, and graft rejection.

Specific pathogen recognition by CD4+ T cells occurs through the TCR and an interaction with a peptide/MHC class II complex. A tightly regulated event, TCR activation results in a signaling cascade leading to proliferation, differentiation or even apoptosis (72). The different subsets of CD4+ T cells include the interferon-gamma (IFN-γ) producing Th1 cells, the IL-4 producing Th2 cells responsible for regulation of humoral immunity, and the newly emerging Th17 "pathogenic" T cells (73). CD8+ T cells recognize endogenous antigens presented on MHC class I molecules found on all nucleated cells in the body, and are responsible for the clearance of infected cells.

The T cell Receptor (TCR) signaling cascade

The TCR on resting T cells is comprised of an αβ heterodimer and is associated with the δ,γ,ε, and ζ chains to for the TCR/CD3 complex (74). Following engagement of the TCR, a well defined signaling cascade is activated. The cytoplasmic domains of the TCR contain immunoreceptor tyrosine-based activation motifs, or ITAMs, which are activated by phosphorylation of specific tyrosine residues by the src family kinase members Lck and Fyn (75). The Syk family member ZAP70 is recruited to the TCR/CD3 complex through interaction with the phosphorylated ITAM motifs, followed by interaction with and activation of the membrane adaptor molecule LAT (linker for activation of T cells) and SLP-76 (SH2-domian containing leukocyte protein of 76 kDa). Together, these later components nucleate a complex that includes the proteins Grb2, Vav-1, the p85 regulatory component of PI3K, Sos (son of sevenless), PLCγ1

(phospholipase Cγ1) , and GADS (Grb2-related adapter downstream of Shc) (reviewed in

(76). The major downstream target of this multisubunit complex is IP3 (inositol-1,4,5-

triphosphate) and DAG (diacylglycerol), which is hydrolyzed from PIP2

(phoshatidylinositol 4,5 bisphosphate) mediated by the actions of PLCγ-1. Immediately

following IP3 production, calcium stored in the endoplasmic reticulum (ER) is released

through the CRAC channels, and the increase in intracellular Ca^{2+} levels activates the

Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin. Activated

calcineurin then dephosphorylates the transcription factor NFAT (nuclear factor of

activated T cells) which localizes to the nucleus to regulate a variety of genes.

The other component of that cascade, DAG, requires PLCγ-1 to directly target

another serine/threonine kinase found associated with the TCR/CD3 complex, PKCθ

(protein kinase C). PKCθ when activated at the TCR interacts with a multisubunit

complex called the CBM which contains CARMA1, Bcl10, and MALT1 (77). This activated

CBM complex is required for IKK activation, which subsequently phosphorylates IκBα

leading to the release and nuclear import of NF-κB (78). These two pathways of

activating the transcription factors NFAT and NF-κB are the characteristic signaling

products as a result of T cell activation, but are among multiple factors that lead to, and

are required for, optimal T cell activation.

TCR stimulation alone is insufficient to activate a T cell to proliferate. In fact, TCR

stimulation without costimulation results in T cell unresponsiveness and anergy (79).

Costimulation of TCR with CD28 is required for optimal T cell activation and *IL-2* gene

expression, and studies *in vivo* have shown that blockade of CD28 leads to T cell anergy (80). The ligands for CD28 are B7.1 and B7.2 (CD80 and CD86) and are expressed and upregulated on APCs following CD40-CD40L interaction between T cell and APC. The signaling events following CD28 ligation by B7.1 and B7.2 requires similar adaptor and signaling molecules utilized by the TCR/CD3 pathway, thus the CD28-mediated signal is an enhancing signal and a threshold requirement of T cell activation (81). In contrast, CTLA-4 is an inhibitory molecule found on activated T cells that has homology to CD28 and a higher affinity for B7 receptors (82). Thus, CTLA-4 acts to block CD28 receptor engagement and acts as an inhibitory receptor. Other TCR costimulatory (B7h/ICOS)(83) and inhibitory (PDL-1/PD1)(84) receptor partners work together to regulate TCR activation and subsequent gene expression in T cells.

Part IV: *IL-2* promoter regulation- A model for T cell activation requirements

Transcriptional regulation at the *IL-2* promoter: the promoter defined

Initial work to determine the regulatory elements of the *IL-2* promoter characterized an approximately 300 base pair sequence upstream of the TATA box in both murine and human T cells that was sufficient to drive the expression of a reporter gene in *in vitro* transfection experiments (85, 86). Studies done in transgenic mice expressing only 500 bases of the proximal sequence of the *IL-2* promoter, however, was unable to control positional effects of integration and tissue specificity of transcription.

Therefore more distal regions of *IL-2* are required for full promoter regulation (87, 88). But for all intents and purposes, the 300 base pair region (proximal promoter) originally described encodes the transcription factor binding domains absolutely required for *IL-2* expression. Since the early 1990's, the transcription factors that bind to this minimal promoter have been intensely studied and described, and the following section will go into depth on the multiple factors and mechanisms of *IL-2* gene expression with a focus on the proximal enhancer/promoter region.

Transcriptional regulation at the *IL-2* promoter: Transcription factors.

NF-κB- The transcription factor NF-κB plays a large role in several aspects of T cell function, including development, homeostasis, activation, survival and function. The importance of NF-κB is underscored by the effects of aberrant activities of NF-κB resulting in autoimmunity, cancer, and inflammation (89, 90). NF-κB is a transcription factor representing a conserved group of proteins in the Rel family, which includes the members p65 (RelA), RelB, p50 (NF-κB1), p52 (NF-κB2) and c-Rel. The Rel family can be separated into two distinct groups; (i) the RelA, RelB and c-Rel molecules are characterized by the conserved rel-homology domain and contain a transactivation domain; (ii) whereas the second group consists of the remaining p50 and p52 molecules which lack a transcriptional activation domain and are processed from large precursor proteins p105 and p100, respectively (91). NF-κB is found dimerized in the cytoplasm retained by IκB's, a family which contains seven members which include IκBα, IκBβ, IκBε,

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IκBγ, Bcl3, p100 and p105 precursors (92). The predominant dimers of NF-κB found in T

cells includes the p50 homodimer, the p50/c-Rel heterodimer, the p65/p50

heterodimer, the latter two playing a substantial role in *IL-2* transcriptional regulation

(93). As stated previously, activatin of TCR leads to a signaling cascade resulting in the

activation of the IKK complex, proteosomal destruction of IκB followed by NF-κB

translocation to the nucleus and activation of *IL-2*. Although NF-κB is required for the

optimal expression of *IL-2*, other factors play an equal if not more important role in

transcriptional regulation.

      NFAT/AP-1- The NFAT family of proteins plays a fundamental role in cytokine

gene regulation in T cells. This family contains five members which include NFAT1

(NFATc2 or NFATp) which is found at high levels in resting T cells (94), NFAT2 (NFATc1 or

NFATc) is induced following activation, NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5.

These members have a high sequence homology in the DNA-binding domains (DBD),

which interestingly also have homology to the Rel/NF-κB DBD (95, 96). In T cells and

most notably at the *IL-2* promoter, NFAT requires the concomitant binding of AP-1 for

activity. AP-1 binds the promoter in close proximity to at least three sites, with NFAT at

two distinct sites, with Oct-1, and potentially two additional NFAT sites (97-100). As

previously outline, the activation of NFAT and AP-1 are both calcium-dependent,

entering the nucleus following TCR stimulation. There are other transcription factors

necessary for complete *IL-2* expression, such as Oct-1, NAB2, Bcl11B and others, but

they are out of the scope of this introduction. Remarkably, the inhibition of any one of

the above transcription factors ablates *IL-2* expression even though other factors are

still abundant. This interesting notion brings us to the concept of the enhanceosome in *IL-2* gene regulation.

The *IL-2* enhanceosome

One interesting model generated from the work of Rothenberg *et al.* is the concept of an enhanceosome-like mechanism to regulate *IL-2* (93). The concept of the enhanceosome was first modeled after the viral infection-inducible responsive gene *IFN-β* (101, 102). The concept follows the idea that an enhancer works as an integration platform, where specific factors triggered by specific responses must be available in an all or nothing assembly. In the case of the *INF-β* enhanceosome, the high mobility group protein HMGI(Y) is required to bind the promoter/enhancer region, and any mutation in this site or other surrounding transcription factor binding sites nearly ablates all *in vivo* activity (103). Similarly, at the *IL-2* promoter, inhibition of one factor, such as NFAT, AP-1 or NF-κB, single handedly inhibits gene activation. A clear example of this occurs during anergy induction, wherein TCR activation without CD28 costimulation results in T cell unresponsiveness and no *IL-2* induction. Even more striking, Rothenberg goes on to demonstrate through *in vivo* footprint analysis that a lack of DNA-protein interaction is evident at the proximal promoter in unstimulated T cells, and only under optimal stimulation conditions does a DNA-protein footprint appear. Addition of a calcium (NFAT) or NF-κB inhibitor, such as Cyclosporin A or forskolin, to an activated T cell results in dissolution of the footprint completely. Thus, the enhanceosome-like complex

found at the *IL-2* promoter is exceptionally unstable, requiring optimal signals and abundance of multiple transcription factors for activation. Later sections will focus on the step preceding the binding of transcription factors to the *IL-2* promoter, that is to say the role of chromatin remodeling required for gene expression.

Transcriptional regulation of the HIV LTR: Lessons learned from *IL-2*?

HIV infects multiple cell types, from macrophages to lymphocytes, thymocytes, dendritic cells among others. The ability of HIV to adapt to each of these cell types is apparent in studies comparing the capacity of HIV gene transcription in different cell types. Unlike the above description of the enhanceosome-like complexity of the *IL-2* promoter, the HIV promoter only requires a few specific factors for optimal transcription. Importantly, the transcription of HIV-1 LTR following integration into the genome occurs in two steps, (i) the early phase requires host transcription factors for initiation of transcription, and (ii) the late phase transcription which requires the HIV-1-encoded viral protein Tat (104). The basic architecture of the HIV LTR is divided into 3 regions, the U3 (nucleotides -453 to -1), R (+1 to +98) and U5 (+99 to +180), which contains the regulatory element (-453 to -104), enhancer (-105 to -79) and core domains (-78 to -1) (all found in the U3) along with the TAR regulatory domain (+1 to +60) found in the R region (105). The basal transcription elements of HIV LTR required in all cell types resides in the core promoter region, and includes the TATA box for binding of host transcriptional machinery and the adjacent Sp1 transcription factor binding sites (106,

107). Sp1 is able to recruit or synergize with activation factors such as COUP-TF (108), NF-κB (109), and Tat (110), and has been reported to recruit CyclinT1 for Tat-independent transcription (111). The importance of this region is underscored by the fact that deletion of the core promoter ablates LTR transcription in all cells types.

The enhancer region described previously contains two adjacent NF-κB binding sites with an overlapping NFAT binding site. The NF-κB sites are critical for optimal LTR activity. Similar to the *IL-2* promoter, the most abundant NF-κB factor found in activated T cells, the p65/p50 heterodimer, is largely responsible for binding to and activating the LTR. Deletion of the NF-κB binding sites greatly reduces the ability of viral replication in CD4+ T cells and other cell types as well. An interesting and not fully understood interplay also occurs in this region. The NFAT binding site overlaps the NF-κB binding site, and therefore based on stearic hindrance, binding of these two factors are mutually exclusive at this site. The importance of NFAT in LTR transcription is supported by the observation that NF-κB and Sp-1 mutant viruses replicate in CD4+ T cells upon phorbol ester and Ionomycin stimulation. NFAT has been shown to be both a positive and a negative regulator of LTR transcription. Specifically, NFAT1 has been shown to be a transcriptional activator and repressor in CD4+ T cells (112-114), while NFAT2 has been shown to only activate the LTR (115). It is not all together clear the role that the different NFAT and NF-κB factors play in binding to the LTR, but differential abundance of each factor in unstimulated CD4+ T cells and stimulated cells is intriguing. As noted earlier, there is an abundance of NFAT1 in unstimulated cells, and NFAT2 levels rise sharply in an activation-dependent manner. One could imagine a situation where

NFAT1 would be inhibitory to LTR expression in naïve T cells, whereas NFAT2 might be a more potent activator of LTR and is abundant in stimulated T cells. Thus it is intriguing to speculate that HIV-1 LTR has adapted to use most efficiently the transcription factors that are available under conditions favorable for replication. This theory is supported by Mouzaki *et al*. when they described a repression-derepression mechanism in primary T cells that is NFAT dependent (116). Although NFAT and NF-κB are the two key enhancer molecules that have been heavily studied and described, a region just upstream of the enhancer contains a regulatory element capable of binding multiple factors in a cell and environment specific way, and a comprehensive review can be found elsewhere and thus is not a focus of this review (104).

One keys aspect of HIV replication is the innate ability of the virus to adapt to multiple cellular environments, both as an efficient way of replication and evasion of immune responses. Not so surprisingly, the LTR has also evolved to exploit the mechanisms of the host cell, and thus has an exquisite ability to respond to host cell stimuli. With respect to the HIV LTR, the *IL-2* promoter acts as a model of activation-dependent regulation of gene transcription. The following chapters will focus on another important method of promoter regulation, focusing on chromatin structure and factors responsible for regulation of chromatin remodeling at both the *IL-2* and LTR promoters.

Part V: Chromatin remodeling

Chromatin remodeling at the *IL-2* promoter

Insights into the regulation of the *IL-2* promoter were provided by studies determining the tissue specificity of *IL-2* expression. So far, tissue-specific transcription factors have yet to be revealed, leading investigators to search for the possible mechanism of promoter regulation by chromatin modification. Early studies by Rothenberg's group demonstrated the dynamics of cis-regulatory regions in the first 600 base pairs upstream of the transcription start site. In the 5' region flanking the proximal promoter, approximately -510 to -313, is maintained in an open chromatin conformation in both unstimulated and stimulated CD4+ T cells (117). This is in stark contrast to the proximal promoter, -300 to -45, which is restructured within 1-2 hours following antigenic stimulation (118). More importantly, the distal promoter region maintains DNA/protein interactions, suggesting a role of this region in nucleosome remodeling. In 2001, Attema *et al.* described a positioned nucleosome located from -200 to -60, which following activation, is remodeled and associated with loss of histones H3 and H4 and a decrease in histone acetylation (119, 120). Thus, the current model of histone dynamics and chromatin remodeling at the *IL-2* gene is a regulatory complex positioned just upstream of the proximal promoter that contains bound factors in unstimulated T cells. Upon stimulation, these factors facilitate the destabilization of the nucleosome positioned at the proximal promoter, followed by binding of transcription factors required for optimal *IL-2* transcription. It is currently unknown which factors

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contribute to or are required for chromatin accessibility at the *IL-2* promoter, but the role of ATP-dependent chromatin remodeling factors is likely required.

ATP-dependent chromatin remodeling complexes

In eukaryotic cells, histone modifying enzymes and also ATP-dependent chromatin modifying complexes play a crucial role in unraveling or relaxing tightly bound chromatin to allow access to transcription factors (121, 122). Enzymes such as histone acetyltransferases (HAT) and histone deacetylases (HDAC) modify specific residues on histone tails, and other molecules are known to regulate the phosphorylation, methylation and ubiquitination as ways of tightly regulating histone dynamics (122-124). While covalent modifications actively repress or activate transcription of various promoters, other mechanisms of chromatin remodeling are in place, including DNA methylation, the use of histone variants, and histone displacement. The latter will be the focus of this section, wherein we discuss the mechanism of histone displacement and the requirement of ATP-dependent chromatin remodeling complexes.

The nucleosome consists of 146 base pairs of DNA wrapped twice around a histone octamer containing two copies of H2A-H2B and H3-H4 dimer pairs, which can be further packaged with linker histones (H1) to create higher order structures. The exact mechanism of nucleosome remodeling remains elusive, and "remodeling" refers to the process of altering the DNA-histone interaction in a process requirimg ATP hydrolysis. Several models of nucleosome remodeling exist, including sliding, twisting and looping,

and these are reviewed in Cosgrove *et al.* (125)*.* The result is chromatin that has been

freed from nucleosomal interaction, allowing for other factors to bind. Four members

of ATP-dependent chromatin remodelers responsible for maintaining this chromatin

fluidity includes SWI/SNF (switching defecting/sucrose non-fermenting), ISWI (imitation

SWI), the INO80 (inositol requiring 80) and the Mi-2/NuRD (nucleosome remodeling and

deacetylation) complexes. We will focus on the latter in the following section.

The Mi-2/NuRD complex: Dynamic regulator of transcription.

The ATPase containing molecule of the Mi-2/NuRD complex is Mi-2β (CHD4).

The CHD family, a subclass of the SWI/SNF family, contains CHD1-5 and are

characterized by conserved ATPase domain, PHD finger domain, chromodomains and a

putative DNA-binding domain (126). The two most widely studied CHD members, CHD3

and CDH4 (Mi-2α/β) were initially identified as autoantigens found in dermatomyositis,

a disease of the connective tissue (127, 128). As its name states, the NuRD complex is

high molecular weight complex that bridges two fundamental actions of chromatin

remodeling, ATPase activity and histone deacetylation. Associated with the Mi-2/NuRD

complex are HDAC1 and HDAC2 which are responsible for the main deacetylase

function, the H4 interacting proteins RbAp46/48, methyl-CpG binding molecules

MBD2/3, and the metastasis associated (MTA) family members MTA1-3 (129-132).

Mi-2β is the main subunit containing the ATPase activity. By itself it can interact

with chromatin but not free DNA or histone, and the ATPase function is required in

nucleosome displacement (133, 134). As stated previously, the reported HDAC function of the Mi-2/NuRD complex has been shown to be critical for the repression of a multitude of promoters. Even though Mi-2β contains a putative DNA binding domain, it can be targeted to promoters through the interaction with transcription factors such as Bcl6, ikaros and Bcl11B (135-137). Interestingly, the first loss-of-function study described a role for Mi-2β in the activation of the *CD4* gene in developing thymocytes. This was the first study to show that Mi-2β was able to act as an activating complex through its association and recruitment of p300 HAT (138). Other groups have shown that Mi-2β can act as an activator or repressor of transcription depending on the association of Mi-2β with RET Finger Protein (RFP) and Brg1 biochemically (139). Thus, ATP-dependent chromatin remodeling factors are required for differential regulation of gene transcription by multiple mechanisms.

So far, the molecules responsible for increasing the chromatin accessibility at the proximal *IL-2* promoter following activation have not been described. The role Mi-2β at the *IL-2* promoter has not been previously determined, although several factors that are known to associate with the Mi-2/NuRD complex, such as NAB2 and Bcl11B, are factors required for the activation of the *IL-2* promoter *in vivo* (137, 140-142). More importantly, these ATP-dependent chromatin remodeling factors are important in the regulation of *IL-2* in regulatory T cells. The fact that murine Treg cells fail to undergo proper chromatin remodeling across the proximal *IL-2* promoter supports the role of chromatin remodeling factors in gene regulation (143).

Chromatin remodeling at the LTR

Transcription of the HIV LTR follows integration into the target chromosome, with a preference for regions of active replication (144, 145). The chromatin structure of the LTR has been extensively studied for the prospect of understanding how viral latency is regulated. Two nucleosomes are positioned on the LTR, nuc-0 is bound to the Regulatory Element in the U3 region encompassing -415 to -255, while nuc-1 is found in the Tar Element located in the +10 to +155 region of the R domain (146-148). Upon T cell stimulation, nuc-1 is known to actively remodel, and histone acetylation is known to regulate LTR transcription (149, 150). The HAT p300 is recruited by p65 interaction to acetylate histones and activate the LTR (151). Under conditions of viral latency, such as resting or unstimulated cells, the LTR is occupied by a p50 homodimer associated with HDAC1 (152). Similarly, YY1 and LSF have also been shown to recruit HDACs to maintain a hypoacetylation state of histones (153). It is clear that there are multiple levels of regulation of both the *IL-2* and LTR promoters including both transcription factors and chromatin remodeling. The next section will be devoted to the role of HDACs in promoter regulation.

Part VI: Role of Histone deacetylases (HDACs) in T cell and HIV gene expression

General overview of HDACs and HDAC inhibitors

HDACs are the enzymes that mediate the removal of acetyl groups from the ε-amino groups of lysine residues. Originally named for their role in the removal of acetyl groups from histones, it is well known that other proteins are also deacetylated by HDACs. The HDAC family has 18 total members separated into groups based on phylogenetic analysis and homology with yeast HDACs (154). Class I HDACs includes HDAC1-3 and 8, class II is comprised of group IIa which includes HDAC4,5,7 and 9, and IIb containing HDAC6 and 10. Class III contains SIRT1-7, and class IV contains HDAC11. In general, class I HDACs remain nuclear while the class II HDACs are able to shuttle between the nucleus and cytoplasm. HDACs have become extremely promising therapeutic targets due to their aberrant expression and association with cancer (reviewed in (155)), and HDAC inhibitors (HDACi) have shown therapeutic potential in several hematologic malignancies (156-158).

The most extensively studied HDACs are the class I and II proteins. All HDACs in these two classes contain similar catalytic domains, the HDAC domain. Unlike the class I HDACs, class II HDAC contain an extended amino-terminal adapter domain that has been shown to interact with several transcription factors (reviewed in (159)). The contribution of the catalytic domain of the class II HDACs in transcriptional repression has been disputed. Attempts to successfully isolate recombinant forms of class II HDACs with enzymatic activity has been elusive (160, 161), and natural splice variants of HDAC9 lacking the HDAC catalytic domain or overexpression of the N-terminus alone is able to associate with full HDAC activity (162, 163). More recently, Lahm *et al.* demonstrated evidence that class II HDACs contain a conserved histidine in the catalytic domain that is

different from class I HDACs, which instead contain a tyrosine at this site. Swapping the

histidine 976 for a tyrosine conferred class I enzymatic levels to class II enzymes, and

vice versa. Thus, class II HDACs intrinsically contain low deacetylase activity and require

associated class I molecules *in vivo* (164).

Biological role of HDACi

HDACi in treatment of cancer has been shown to selectively kill tumor cells,

which are approximately tenfold more sensitive to HDACi induced apoptosis (165).

Though the mechanism is not completely understood, evidence of apoptosis induction

through death receptor extrinsic pathways, mitochondrial pathways, reactive oxygen

species (ROS) production, and cell cycle arrest have all been described (reviewed in

(166)). There is increasing evidence for a role of HDACi in immunomodulation, the

enhancement of anti-tumor immune targeting. Groups have shown that HDACi

upregulates MHC class I and II molecules, costimulatory molecules such as CD40, CD80,

CD86 and ICAM1 to augment the immunogenicity of tumor cells (167, 168). The HDACi

suberoylanalide hydroxamic acid (SAHA) can suppress acute graft-versus-host disease

(GVHD) through modulation of cytokine production (169). Recently, the Hancock group

determined that *in vivo* administration of the HDACi TSA along with rapamycin was able

to enhance Treg-mediated suppression of inflammatory bowel disease, induce islet

allograft survival and donor-specific allograft tolerance. In this study, TSA enhanced the

expression of *Foxp3*, and increased Treg numbers and enhanced Treg suppressive

activity on a per cell basis (170). Taken together, HDACs play a large role in immune

regulation and cellular activity.

HDACs and HDACi on the *IL-2* promoter

As stated in the previous sections, HDACs play an extensive role in gene

regulation in immune cells. Approximately 2-8% of gene are enhanced or repressed by

HDACi (171-174), and in a recent study of HDAC1-specific knockdown in embryonic stem

cells, approximately 7% of the genes were dysregulated (175). Surprisingly, a number of

genes were enhanced or inhibited by HDAC1 knockdown or HDACi, due to both direct

effects and indirect effects. In T cells, *IL-2* transcription has been shown to be

downregulated upon HDACi treatment, although the specific HDAC targeted by HDACi

administration was not investigated (176-178). One of the downstream targets of

HDACi is NF-κB. Specifically, it has been shown that HDACi can alter the transactivation

activity of NF-κB without altering nuclear localization or DNA binding activity. Still,

others have shown that HDACi can enhance NF-κB nuclear localization, and there is

evidence that HDACi can differentially regulate NF-κB in a cell-dependent manner.

Recently, Matsuoka *et al.* demonstrated HDACi disrupts the HDAC4/N-CoR complex as a

mechanism of *IL-2* repression (179), thus class II HDACs can regulate *IL-2*. It remains to

be examined if class I HDACs can regulate *IL-2* transcription.

HDACs and HDACi on HIV LTR

LTR regulation by HDACi has been described by multiple groups using TSA, trapoxin (TPX), valproic acid (VPA) and sodium butyrate (NaBut). The addition of these inhibitors leads to an increase in nuc-1 acetylation and thus transcriptional activation of LTR that are transiently transfected, stably integrated and in the context of reconstituted chromatin templates (150, 180-186). The viral protein Tat is hyperacetylated upon HDACi treatment leading to more efficient transcriptional activity. Similar to *IL*-2, regulation of p65 by deacetylation affects the activity of HIV LTR. Removal of the NF-κB elements inhibited the effects of HDACi on the LTR (183). Interestingly, addition of TSA or NaBut resulted in the prolonged nuclear localization of p65/p50, and also delayed the reappearance of cytoplasmic IκB through an IKK-dependent activation. Therefore, HDACi is able to affect the activity of NF-κB not only through regulation of acetylation, but also through upstream mediators of the NF-κB pathway.

Regulation of HDACs

HDACs play important roles in multiple processes of transcriptional regulation and are therefore tightly regulated. Several HDACs are found in large multiprotein complexes and are thus regulated by association with cofactors. As previously stated, purified recombinant class II HDACs are enzymatically inactive, thus association with *in vivo* binding partners will regulate HDAC activity. HDAC1 and HDAC2 are found in at least three different multisubunit complexes, including the Mi-2/NuRD (see previous

section), Sin3 and CoREST complexes (129, 132, 187-189). Separate from complex

formation, HDACs can also be covalently modified *in vivo*. Several groups have

demonstrated phosphorylation of HDAC1 and HDAC2 at multiple sites, and it appears as

though the extent of phosphorylation results in differential HDAC enzymatic activity

(190-192). Others have shown that HDAC1 can be modified by sumoylation at lysine's

444 and 476, although the functional activity following modification remains to be

clarified (193, 194). Interestingly, there have been multiple reports of viral proteins

regulating HDACs through association *in vivo*. The Adenoviral gene product Gam1

inhibits HDAC1 deacetylase activity through changes in sumoylation (194), and CMV

immediate early 1 (IE1) and IE2 proteins are able to inhibit HDAC3 through unknown

mechanisms (195).

Part VII: Foxp3 Regulation of gene transcription

***Foxp3* gene expression signature- a 'master regulator'?**

The notion of Foxp3 as a "master regulator" or "lineage commitment" factor has

been scrutinized and the role of Foxp3 in gene regulation has been extensively studied.

The hallmark of a lineage factor is that the presence or absence of the factor is sufficient

to confer or preclude a particular phenotype. Such a role for Foxp3 was identified on

the basis that loss of function results in severe immunodeficiency, and that retroviral

transduction of Foxp3 restored suppressive ability to a degree (33, 34). Evidence that

Foxp3 might not be absolutely required for Treg function exists. Nearly all human T cells contain some level of Foxp3 following activation without any obvious suppressive activity (196, 197). In human IPEX patients, a select population of T cells with regulatory activity was able to persist in the absence of *Foxp3* gene expression (198). Consistent with this notion, several groups have also shown T cells with characteristics of Treg cells in the periphery of mice in the absence of *Foxp3* (199, 200). Hill *et al.* extensively looked at the gene signature of Treg cells, both natural and induced under several conditions, and concluded that Foxp3 is perhaps one of several factors required for the full phenotype of a Treg cell (201). Studies utilizing transgenic mice with GFP coding sequence inserted into the *Foxp3* locus, GFP+ Foxp3-less mice, showed that several features of Treg cells are maintained in GFP+ Foxp3-less cells (199). The current hypothesis is that Foxp3 stabilizes or amplifies features of chronic TCR stimulation, although several features of Treg function, such as suppression and cell cycle progression, are highly dependent on Foxp3 (201, 202). In this regard, genes such as *Ctla4, GzmB* and others required for negative regulation of T cell signaling are further amplified by *Foxp3* expression or reduced in GFP+ Foxp3-less cells. Foxp3 is also able to override other developmental T cell pathways, such as Th17, Th1 and Th2 programs, demonstrated by the increased expression of related cytokines in the absence of Foxp3 (199, 203-205). Importantly, the level of Foxp3 expression is crucial to Treg function. FILIG mice, which harbor a mutation resulting in low expression levels of Foxp3, show a accelerated onset of autoimmune disease (204). Similarly, transient expression of Foxp3 in humans occurs in most all T cells, and only the high expressing Tregs are suppressive.

Regardless, Foxp3 is required for a set of critical functions of Treg cells, and the

mechanisms of Foxp3-mediated gene regulation will be reviewed in the following

sections.

Foxp3 protein structural analysis

In order to understand the function of Foxp3 in transcriptional regulation, the

organization, domains and binding partners must be identified and characterized. The

Foxp3 protein is approximately a 47kDa protein containing 431 amino acids in humans

and 429 in mice with approximately 86% homology. Foxp3 protein is characterized by 4

functional domains; the N-terminus contains a proline-rich region contained in amino

acids 1-193, a C2H2 zinc-finger motif (200-223), a leucine-zipper-like motif (240-261)

and a carboxy-terminal forkhead domain (338-421). Insight into the functional role of

these domains is demonstrated in IPEX patients, where natural mutations of *FOXP3*

have been described for every domain except the zinc-finger.   To date, 13 mutations

have been described in IPEX patients. A mutation in the proline-rich region, del 249 (T)

in exon 2, results in a frameshift and early termination (206). Two mutations are found

in the leucine zipper region, both of which are codon deletions (del770-772 and del772-

774) in exon 7 (207, 208). The Forkhead domain contains 5 separate missense

mutations, R347H, I363V, F371C, A384T and R397W (206, 208-210). Other mutations

include mutations in the stop codon, resulting in C-terminal extensions, a splice site

mutation in the 5' end of exon 5, and an A to G transition at position +4 in intron 8

causing the deletion of exon 9 (207, 209-211). Studies with various IPEX mutants have demonstrated multiple roles of these domains in Foxp3 function. The leucine zipper has been show to be required for oligomerization *in vivo*, wherein deletion of amino acids 250 or 251 results in a loss of Foxp3 homotetramerization or Foxp1/Foxp3 heteromerization (212-214). Interestingly, mutations in the leucine zipper domain disrupt binding and transcriptional function, but does not impair association of Foxp3 with a multiprotein complex. Analysis by the Ziegler group demonstrated that the N-terminus and forkhead domain are required for repression of targeted promoters, with the proline-rich region alone capable of repression, and the forkhead domain required for DNA binding and nuclear localization. Also, while disruption of the leucine zipper was necessary and sufficient to abrogate multimerization, a mutation disrupting the zinc finger of Foxp3 (C204S) had no effect on Foxp3 multimerization and had only a mild effect on repression of a targeted promoter (214).

Regulation of Foxp3 protein

Currently two isoforms of Foxp3 are found in human Treg cells, a full length transcript and an exon 2 deleted form (215, 216). Murine Foxp3 protein is only expressed in the full length form and found to be completely nuclear (217). Conversely, the full length human Foxp3 (Foxp3a) is found both in the cytoplasm and nucleus while the truncated isoform (Foxp3b) is nuclear only due to a lack of a putative nuclear export signal (NES) contained in this region (218). Induction of *Foxp3* gene expression in Treg

cells is currently being studied. Several groups have documented the ability of TGF-β to convert T cells into Treg cells (219, 220), and recent investigation of the Foxp3 promoter has demonstrated a dependence of NFAT, Sp1, AP-1, STAT5 and, more recently, Smad3 for *Foxp3* activation (221-223). While the factors regulating *Foxp3* expression are under intense investigation, a more thorough look into the temporal regulation of Foxp3 gene expression and the transcription factors required will give insight into the requirements for Treg development, function and maintenance.

Mechanisms of Foxp3 regulation of promoter activity

Extensive studies on the gene profile of Treg cells along with ChIP-Chip (microarray on immunoprecipitated chromatin) experiments determining the direct targets of Foxp3 protein has produced insight into the requirement of Foxp3 protein for the Treg signature. It is now evident that multiple signals, including TGF-β signaling, IL-2 receptor signaling and Foxp3 converge to impart this Treg signature. Nevertheless, there are multiple characteristic promoters that are directly regulated by Foxp3, including *IL-2, Ctla4, Tnfrsf18 (GITR)* among others. The mechanisms of Foxp3 regulation of these promoters will be discussed in the following paragraph.

Foxp3 was initially described by Bettelli *et al.* to functionally interact with and inhibit the transactivation activity of the transcription factors NFAT and NF-κB (224). More biochemical and structural analysis of the interaction of Foxp3 and NFAT was described by Wu *et al.*, wherein mutations in the forkhead domain abrogating NFAT and

Foxp3 interaction was able to inhibit Foxp3 promoter occupancy and regulation of several characteristic genes. Thus, a model was proposed where Foxp3 was able to inhibit an activating NFAT:AP-1 complex by promoting a repressive NFAT:Foxp3 complex at the *IL-2* promoter (225). More recently, Foxp3 was also demonstrated to inhibit AP-1 DNA binding activity to further inhibit AP-1-dependent genes such as *IL-2* (226). Reports from Grant *et al.* and our published data point to a role of Foxp3 in transcriptional regulation of retroviral promoters. In both cases, the HIV-1 promoter was regulated by Foxp3-mediated modulation of NF-κB, although with disparate results (68, 69). Interestingly, two studies showed an increase or 'stabilization' of transcription factors at various promoters regulated by Foxp3, correlating with the notion that Foxp3 might play a role of enhancing or stabilizing a TCR-mediated signal (68, 225). Factors involved in T cell development also interact with Foxp3 to regulate targeted promoters. The Sakaguchi group described an interaction with Foxp3 and AML1/Runx1 transcription factor. In this study, AML1/Runx1 bound to a region upstream of the core enhancer region of the *IL-2* promoter and enhanced *IL-2* expression. A Foxp3/AML1 complex was able to bind this region and was shown to be required for Foxp3 repression of *IL-2* (227).

Foxp3 regulates the expression of various promoters at multiple levels. Several groups have demonstrated an increase or decrease in histone acetylation of promoters regulated by Foxp3 (68, 225, 228). In accordance with this notion, factors modulating histone acetylation, such as HATs and HDACs, have been shown to interact with and be recruited by Foxp3 to various promoters. Li *et al.* describes an ensemble of Tip60 (HAT), HDAC7 and Foxp3 in the regulation of *IL-2*. They demonstrated that the presence of

these three factors together optimally suppressed gene expression, and knockdown of

any one of these factors inhibited Foxp3 repression of *IL-2* (229). More recently, work

from our lab and others has shown an interaction of Foxp3 with linker histone H1b

(Mackey-Cushman manuscript in preparation) and histone H1/H5(230), respectively,

which are required for Foxp3 regulation and suppressive function. Thus, it is becoming

abundantly clear that Foxp3 regulates multiple T cell pathways and we are just now

beginning to unravel the complex nature of Foxp3 gene regulation in Treg cells.

Scope of this dissertation

This dissertation is encompasses the emerging role of Foxp3 in multiple aspects

of the immune system. The purpose of the following chapters is to characterize several

mechanisms of Foxp3 function. The first chapter will demonstrate a disparate role for

Foxp3 protein in the regulation of the *IL-2* promoter and the HIV LTR in human Treg

cells. Through a yet unknown mechanism, Foxp3 activates the HIV-1 LTR by stabilizing

the transcription factor NF-κB bound to the HIV promoter. The second chapter focuses

on a novel function of Foxp3 in Treg cells. Here, we demonstrate that Foxp3 inhibits the

activity of HDAC1 in human T cells. We also show that this function is important for

promoter regulation, since knockdown of HDAC1 inhibits Foxp3-mediated repression or

activation of various promoters. Finally, the third chapter demonstrates a role for Foxp3

in the regulation of *IL-2* gene expression through chromatin remodeling factors. We

show that the ATPase Mi-2β is required for *IL-2* activation, and expression of Foxp3 is associated with a decrease in Mi-2β binding at the proximal promoter. Herein, we describe multiple novel mechanisms for Foxp3-mediated gene regulation through transcription factor regulation and chromatin remodeling factor modulation. The implications of these findings and future directions will be discussed in the final chapter.

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Chapter II

FoxP3 Enhances HIV-1 Gene Expression by Modulating NF-κB Occupancy at the LTR in Human T Cells

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Abstract

FoxP3 determines the development of CD4+CD25+ regulatory T (Treg) cells and represses IL-2 expression in Treg cells. However, HIV-1 infects and replicates efficiently in FoxP3+ Treg cells. We report that, while inhibiting *IL-2* gene expression, FoxP3 enhances gene expression from HIV-1 LTR. This Foxp3 activity requires both the N-terminal and C-terminal domains, and is inactivated by human IPEX mutations. FoxP3 enhances HIV-1 LTR via its specific NF-κB binding sequences in an NF-κB-dependent fashion in T cells but not in HEK293 cells. FoxP3 decreases level of histone acetylation at the *IL-2* locus but not at the HIV-1 LTR. Although NF-κB nuclear translocation is not altered, FoxP3 enhances NF-κB-p65 binding to HIV-1 LTR. These data suggest that FoxP3 modulates gene expression in a promoter sequence-dependent fashion by modulating chromatin structure and NF-κB activity. HIV-1 LTR has evolved to both highjack the T cell activation pathway for expression and to resist FoxP3-mediated suppression of T cell activation.

Introduction

Although clearly generated in the thymus (1), CD4+CD25+ Treg cells can also be generated from mature T cells in the peripheral organs (2, 3). Recent genetic studies in both mouse and human have identified FoxP3, a Forkhead transcription factor, as a master determinant of Treg development and function (4-7). Mutations in the FoxP3 gene in Scurfy mice or human IPEX patients lead to lymphoproliferative and autoimmune phenotypes due to impaired Treg cells. Importantly, ectopic expression of FoxP3 in naïve CD4+CD25- T cells leads to inhibition of IL-2 expression and Treg-like suppression activity (8). FoxP3+ Treg cells have been implicated in a number of pathologic processes including elevated levels in cancers (9-11) and infectious diseases (12-15), and reduced levels in autoimmune diseases (16-20). With chronic HCV infection in human and chimps (21-23), and friend leukemia virus in mice (14), Treg cells are induced to subdue the anti-viral immune responses and allow persistent infection.

HIV-1 disease or AIDS progression, however, is associated with overt immune activation. Since the beginning of the AIDS epidemic, it has been documented that chronic immune activation is a reliable predictor of AIDS progression (24-26). Interestingly, SIV infection in its native hosts is non-pathogenic, characterized by high SIV replication, limited immune responses, and long-lasting Treg induction (27-29). In HIV-1 infected patients, conflicting findings have been reported regarding Treg cells. FoxP3+ Treg cells are generally depleted in parallel to the total CD4+ T cell population

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during disease progression (30). In some reports, increased levels of Treg cells are documented in lymphoid tissues (31, 32), whereas others have observed decreased levels of Treg cells and FoxP3 expression in peripheral blood (13, 33-35). However, in rhesus monkeys acutely infected with SIV, FoxP3+ Treg cells are induced and are productively infected by SIV (36). Thus, it is not clear how HIV-1 infects and replicates in Treg cells in vivo.

One clear function of FoxP3 is to suppress expression of *IL-2* in Treg cells. Direct FoxP3 binding with NFAT has been reported to prevent its association with AP-1 and contribute to suppression of *IL-2* gene expression (37). Moreover, FoxP3 has been demonstrated to occupy the *IL-2* promoter to inhibit gene expression by altering histone acetylation levels, thus changing the chromatin structure (38). In addition, FoxP3 has been reported to inhibit NF-κB activity in transfected 293 cells or T cells (39, 40); however, others have failed to show such inhibitory effect in human T cells (37). Both NFAT and NF-κB are also involved in regulating HIV-1 gene expression (41). Although NF-κ B activates HIV-1 LTR, the effect of NFAT on LTR is controversial. NFATc1 is reported to upregulate HIV-1 gene expression by binding to the overlapping NF-κ B sites (41), and NFATc2 has also been shown to upregulate HIV-1 replication in primary CD4+ T cells (42). In other reports, however, NFATc2 is shown to competitively bind to the LTR NF-κ B sites and inhibit LTR activity (43, 44).

Given that FoxP3 inhibits T cell activation and expression of cytokines such as IL-2 via inhibiting NFAT and NF-κ B, one surprising recent finding reports that Treg cells

support higher levels of infection by HIV-1 or FIV compared to FoxP3-CD4+ T cells in vitro (13, 45). Two lines of evidence have also indicated that HIV-1 infection and replication in Treg cells may be important *in vivo*. First, recent reports have documented that, although less than 5% of total CD4+ T cells from peripheral blood are CD25+FoxP3+ Treg cells, up to 50% of CD4+ T cells express FoxP3 in mucosal lymphoid organs from HIV-1 (46) or SIV (36) infected human or monkeys, respectively. Therefore, the FoxP3+ Treg cells can provide a significant number of target cells for HIV-1 infection in lymphoid organs. Remarkably, 13% of the FoxP3+ T cells are shown to be productively infected by SIV in the lymphoid organs of acutely infected animals (36). Therefore, FoxP3+ Treg cells are important target cells for HIV-1 infection and replication, at least in mucosal lymphoid tissues during acute infection. Treg induction in HIV-1 infected lymphoid organs may contribute to suppressed anti-HIV immunity and establishment of persistent HIV infection. It is therefore critical to investigate how HIV-1 infects and replicates in these T cells for both virological and immuno-pathogenic reasons.

Here we report that FoxP3 enhances HIV-1 LTR but inhibits *IL-2* promoter activity. This FoxP3 activity requires both the N- and C-terminal domains and maps to the N-terminus (including the Proline Rich Region, PRR) and the C-terminal Forkhead domain and is inactivated by human IPEX mutations. We also demonstrate that FoxP3 enhances HIV-1 LTR via specific NF-κB binding sequences in an NF-κB-dependent fashion. FoxP3 also enhances a minimal promoter with the NF-κB sequences derived from LTR but not from the MHC promoter in Jurkat cells, but represses both the HIV-1

LTR and MHC promoter in 293T cells. Interestingly, FoxP3 reduces the acetylation level

of the *IL-2* promoter but increases acetylation of HIV-1 LTR. Furthermore, FoxP3

enhances NF-κB occupancy at HIV-1 promoter in T cells but does not alter NF-κB nuclear

translocation or levels. The data suggest that FoxP3 modulates gene expression in a

promoter sequence-dependent fashion by modulating chromatin structure and NF-κB

activity. HIV-1 LTR has evolved to both highjack the T cell activation pathway for

expression and to resist FoxP3-mediated suppression of T cell activation.

Experimental Procedures

Plasmids, antibodies and cell lines. FoxP3 was cloned into the HSPG retrovirus vector (47). LTR linker scanning mutants (48), LTR-luc, LTR deletion or point mutants, minimal promoter with LTR 3x-κB-luc (41) were previously reported. Mis-sense mutations identified from IPEX patients (8) (I363V, F371C, A384T and R397W) were introduced in the FoxP3 cDNA by site-directed mutagenesis. Deletion mutants in FoxP3 were generated by PCR amplification to specifically remove the N-terminus (aa 1-189) as in FoxP3-ZFLZFKH, the N-terminus including the Zinc-finger (aa 1-241) as in FoxP3-LZFKH, or the N-terminus including all domains except Forkhead (aa 333-429) as in FoxP3-FKH. MHC-3x-κB-luc and IκB-SR plasmids were provided by A. Baldwin (UNC-Chapel Hill, NC, USA). Jurkat cells were maintained in RPMI 1640 (Gibco BRL), 293T cells were maintained in DMEM and primary human T cells were maintained in Iscoves MEM, supplemented with 10% FBS (Sigma), 2mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml Streptomycin. For T cell activation, mouse anti-CD3 and CD28 (BD Biosciences) and Goat anti-mouse IgG (Caltag) antibodies were used. To determine T cell purity, anti-human CD25-PE (Miltenyi) and CD4-FITC (BD Biosciences) were used. 7AAD (Molecular Probes) was used to determine cell viability.

Retrovirus production and transduction. 293T cells were co-transfected with retroviral vector, VSV-G and gag/pol containing plasmids as previously described (47).

For production of single cycle HIV-Luc reporter virus, 293T cells were transfected with VSV-G and HIV-Luc plasmids by Effectene (Qiagen, Valencia, CA). For retroviral transduction, Jurkat cells were spin-noculated with HSPG control or FoxP3 retrovirus to >95% efficiency as measured by GFP expression (47).

Cell culture and T cell purification and transfection. CD4+ primary T cells were purified from Ficoll separated PBMC by MACS human CD4+ T cell isolation kit (>95% purity, Miltenyi-Biotech, Auburn, CA). CD4+ T cells were activated by anti-CD3/CD28 mAb crosslinked by plate-bound anti-mouse IgG as previously described (49). Briefly, purified cells are stained with anti-CD3 (0.5ug/ml) and anti-CD28 (1ug/ml) and incubated on plated-bound goat anti-mouse IgG. Purified primary CD4+ T cells were transfected by Amaxa nucleofector kit (Amaxa Biosystems, Gaithersburg, MD). Briefly, $5x10^6$ unstimulated CD4+ T cells were transfected with 5μg plasmid DNA (>40% efficiency by GFP expression), and cultured for 48 hours before infection with the VSV-G pseudotyped HIV-luciferase reporter virus and activation by CD3 and CD28 crosslinked with plate-bound IgG. Cells were activated for 48 hours and luciferase was measure by luciferase assay. Alternatively, T cells were transduced with vector or FoxP3 retrovirus (>60% efficiency by GFP) and infected with VSV-G pseudotyped HIV-luciferase virus.

IL-2 measurement by real-time qPCR and ELISA. Amaxa-transfected primary CD4+ T cells were sorted for GFP expression and activated with anti-CD3/CD28 as above. IL-2

mRNA levels were determined following activation. 5×10^3 cells were lysed in Trizol

Reagent (Invitrogen) and column purified (RNeasy, Qiagen). cDNA was generated using

Cells-to-cDNA II kit (Ambion, Austin, TX) and quantitative PCR performed on ABI Prism

7000 (Applied Biosystems) using TaqMan IL-2 probe/primer set (Applied Biosystems).

Each sample was normalized to 18s. To measure IL-2 production in Jurkat T cells,

transduced Jurkat cells were stimulated with PHA and Ionomycin (1uM) for 12 hours

(41) and supernatants were collected. Secreted IL-2 was quantitated by the Human IL-2

ELISA kit (BD Biosciences, San Diego).

Transfection of Jurkat T cells and luciferase assay. Jurkat cells were transfected

using Geneporter transfection reagent (Genlantis, San Diego, CA) and transfection

efficiency was monitored by GFP expression or cotransfection with pAX-βgal reporter

plasmid. Cells were lysed and luciferase expression was determined by Luciferase Assay

System (Promega). Experiments were done in triplicates and repeated at least three

times.

ChIP assays. Jurkat T cells with a stably integrated HIV-1 LTR were transduced

with control or FoxP3-expressing vectors. Sonicated chromatin from the Jurkat cells was

IP'ed with control IgG (IgG) or p65 (a kind gift from Al Baldwin) or anti-AcH3 by

chromatin immunoprecipitation assay kit per manufactures instruction (Upstate,

Millipore). Samples were purified and subject to real-time qPCR analysis. The relative

p65 binding or acetylation of IL-2 or LTR promoter is expressed with ratios of ChIP-specific signal divided by signals from control 10% input chromatins. IL-2 promoter was analyzed with Taqman reagents using the following primers: 5'-cac cta agt gtg tgg gct aat gta ac-3' and 5'-ctg atg act ctt tgg aat ttc ttt aaa cc-3' and a FAM:TAM probe 5'-aga ggg att tca cct aca tcc att cag tca gtc-3'. This amplicon spans -226 to -133 in the human IL-2 promoter and spans the NF-κB and AP-1 sites. HIV-1 LTR was analyzed with SyberGreen reagents (Abgene, UK) using the primers (5'-agc cct cag atc ctg cat ata agc a-3' and 5'- gtt agc ca gaga gct ccc agg ctc a-3') that yield an amplicon spanning -44 to +49.

EMSA. Standard EMSA were performed (50). Briefly, probes containing the NFκB site (GGGATTCCCC) from the MHC gene or HIV-1 LTR were labeled with α^{32}P-dCTP using Klenow. 10µg of nuclear extract was incubated with probe.   For antibody supershift or blocking, 2µg of α-p65, α-p50 or control IgG were added to nuclear extract and labeled probe.

Western Blot. 1×10^6 Jurkat T cells were lysed in 0.5% NP-40 lysis buffer and resolved by 10% SDS-PAGE. Approximately 25µg of proteins were used and FoxP3 was probed with an anti-FoxP3 antiserum (51), α-rabbit–IgG–HRP and visualized by ECL(AmershamP P Biosciences).

Statistical analysis. For statistical analysis, a two-tailed Student t-test is employed where $P<0.05$ is considered significant.

Results

FoxP3 differentially modulates IL-2 and HIV-1 gene expression by inhibiting IL-2 but enhancing LTR promoter activity. To investigate the mechanism of HIV-1 replication in FoxP3+ T cells, we expressed FoxP3 in the human CD4+ T leukemia (Jurkat) cells. When Jurkat cells were infected with VSV-G pseudotyped HIV-1 containing luciferase reporter gene, elevated levels of HIV-1 gene expression were detected in both unstimulated and stimulated FoxP3+ T cells (5-8 fold, Fig. 2.1A). Retrovirus-mediated expression of FoxP3 in Jurkat cells inhibited the expression of *IL-2* upon activation (>6 fold, Fig. 2.1B). To determine if FoxP3 similarly regulates expression of *IL-2* and HIV-1 in primary CD4+ T cells, we expressed FoxP3 in purified primary CD4+ T cells. HIV-1 gene expression was significantly enhanced in Th cells previously transduced with FoxP3-expressing retroviral vector (Fig. 2.1C) or by Amaxa nucleofection (data not shown). In contrast, ectopic expression of FoxP3 in CD4+ T cells efficiently suppressed expression from the endogenous *IL-2* gene (Fig. 2.1D). Thus, FoxP3 inhibits *IL-2* expression but enhances HIV-1 gene expression in human T cells.

To determine if FoxP3 regulates expression of *IL-2* and HIV-1 at the promoter level, we transfected IL-2 or HIV LTR promoter constructs driving the luciferase gene into Jurkat T cells. Similar to NL4-luciferase virus infection, FoxP3 also enhanced gene expression from HIV-LTR (Fig. 2.2A). FoxP3 efficiently suppressed IL-2 gene expression from both the endogenous *IL-2* gene (Fig. 2.1B&D) and from the transfected IL-2

promoter (Fig. 2.2B). The HIV-1 LTR co-transfection experiment was performed in the

absence of Tat. Therefore, HIV-1 Tat is not required for the FoxP3 activity.

The Forkhead domain and N-terminus of FoxP3 are critical for FoxP3-mediated enhancement of HIV-1 LTR. The Forkhead domain and distinct residues in FoxP3 are critical for FoxP3 function as indicated by mutations in the scurfy mouse and in human IPEX patients (8). To define the FoxP3 domains that contribute to this LTR enhancing activity, we generated FoxP3 deletion mutants and transduced Jurkat T cells. Loss of the N-terminus, including the Proline Rich Region, inhibited FoxP3 enhancement (Fig. 2.3A) while expression of the N-terminus alone was not able to enhance LTR activity (data not shown). We also tested the known mis-sense mutations derived from IPEX patients by single site-directed mutagenesis introduction into full length FoxP3. Interestingly, all 4 such mis-sense mutations tested also inactivated the FoxP3 activity in enhancing HIV-1 LTR (Fig. 2.3B). Similar levels of the mutant FoxP3 proteins were expressed (Fig. S2.1). Therefore, the FoxP3 activity in enhancing HIV-1 LTR depends on both the N-terminal domain and critical residues in the forkhead domain which are also critical for the function of the FoxP3 protein in humans.

The two NF-κB sites in the LTR enhancer core are both required for FoxP3 enhancement. We have mapped the cis-acting elements in HIV-1 LTR that respond to FoxP3 by employing the 26 linker scanning mutants (LS1-LS26) of HIV-1 LTR (48). The

mutations in LS20 (-111 to -94) and LS21 (-93 to -76), which inactivate the two NF-κB

binding sites in the LTR enhancer core, significantly reduced response to FoxP3 (Fig.

2.4A). Deletion of the NF-κB/NFAT enhancer core completely abolished LTR response to

FoxP3 (Fig. 2.4B). The two NF-κB sites also overlap with the sites for NFAT binding (41).

Point mutations in either NF-κB site (LTR mκB1 and mκB2) significantly reduced FoxP3-

mediated enhancement while mutations in both sites (mn-κB) led to complete loss of

enhancement by FoxP3 (Fig. 2.4B). Therefore the two NF-κB sites in the LTR enhancer

core are both required for FoxP3 enhancement.

FoxP3 enhances HIV-1 LTR activity in NF-κB sequence- and cell type-specific

fashions. To further elucidate the role of NF-κB, we blocked NF-κB activation using the

IκB super-repressor in T cells. Consistent with results that the NF-κB sites in LTR were

critical for FoxP3-mediated activation, FoxP3 was unable to enhance HIV-1 LTR activity

when NF-κB function was inhibited (Fig. 2.5A). We also determined if FoxP3

enhancement was specific to the LTR-derived NF-κB sequences. A luciferase reporter

gene driven by a minimal promoter with 3 NFκB sites derived from HIV-1 LTR (LTR-3κB-

luc) or from the MHC gene (MHC-3κB-luc) was studied in Jurkat cells. Under the same

experimental conditions, FoxP3 enhanced LTR-3κB-luc expression but not the MHC-3κB-

luc expression in T cells (Fig. 2.5B). Interestingly, both LTR-3κB-luc and MHC-3κB-luc, as

well as the LTR-luc were inhibited by FoxP3 in 293T cells (Fig. 2.5C&D), corroborating

results previously described for FoxP3 in 293T cells (39, 40). Thus, FoxP3 modulates HIV-1 LTR activity via its specific NF-κB sites in T cells.

FoxP3 differentially alters the acetylation level of the *IL-2* promoter and HIV LTR. We tested if FoxP3 affects the chromatin structure at the *IL-2* promoter and the HIV-1 LTR by ChIP assays with anti-acetylated histone H3-K9 (AcH3) antibodies. Relative levels of AcH3 were determined by qPCR of the NF-κB and NFAT/AP-1 region (-226 to -133) in the human *IL-2* promoter after ChIP (Fig. 2.6A). FoxP3 reduced the AcH3 level or the "openness" of the *IL-2* promoter in Jurkat cells either unstimulated or after stimulation. When the stably integrated HIV-1 LTR in the same Jurkat A82 (52) cell was analyzed, we discovered that FoxP3 either had no effect (unstimulated) or increased (stimulated) AcH3 acetylation level at the NF-κB /Sp1 enhancer core sequences of the LTR (1.8-fold, p<0.05, Fig. 2.6B). Thus, FoxP3 reduced the histone acetylation level of the *IL- 2* promoter but had no effect or increased that of the HIV-1 LTR in T cells.

FoxP3 does not alter NFκB nuclear translocation, but modulates NFκB occupancy at the HIV-1 LTR promoter. Given that FoxP3 enhancement is both NF-κB sequence- and cell type-specific, it is unlikely that a simple increase in the nuclear levels of NFκB is involved. Indeed, FoxP3 did not enhance NF-κB nuclear localization in Jurkat cells by Western blot analysis (Fig. 2.7A). Using a consensus NF-κB probe, similar levels of p65/p50 and p50/p50 NF-κB DNA binding activity were detected in FoxP3+ and vector

control cells (Fig. 2.7B). We next wanted to determine if FoxP3 was able to modulate

NF-κB occupancy at the HIV-1 LTR promoter in vivo by chromatin immunoprecipitation

(ChIP) using specific anti-p65 antibodies. In a Jurkat T cell line stably integrated with the

HIV-1 LTR (53), FoxP3 enhanced the association of p65 at the LTR promoter (~2 fold, Fig.

2.7C) but not at the *IL-2* promoter (Fig. 2.7D). Therefore, although NF-κB nuclear

translocation was unchanged, FoxP3 enhanced p65 occupancy specifically at the HIV-1

LTR in T cells.

Discussion

FoxP3 inhibits T cell activation and *IL-2* expression in T cells, but high levels of

HIV-1 or FIV infection are reported in FoxP3+ Treg cells *in vitro* (13, 45, 54). Since HIV-1

LTR activity is closely coupled to T cell activation and *IL-2* gene expression, we studied

how FoxP3 modulates *IL-2* gene expression and HIV-1 replication in T cells. We report

that, while inhibiting *IL-2* promoter, FoxP3 enhances HIV-1 LTR. This FoxP3 activity is

inactivated by human IPEX mutations. FoxP3 enhances HIV-1 LTR via its specific NF-κB

binding sequences in an NF-κB-dependent fashion in T cells but not in HEK293 cells.

FoxP3 decreases level of histone acetylation at the *IL-2* locus but not at the HIV-1 LTR.

Although NF-κB nuclear translocation is not altered, FoxP3 enhances NF-κB binding to

HIV-1 LTR. These data suggest that FoxP3 modulates gene expression in a promoter

sequence-dependent fashion by modulating chromatin structure and NF-κB activity.

HIV-1 LTR has evolved to both highjack the T cell activation pathway for expression and

to resist FoxP3-mediated suppression of T cell activation.

HIV-1 infection of Treg cells is well-supported by a number of studies *in vitro* and

in animal models *in vivo*. In fact, HIV-1 (or FIV) can replicate more efficiently in human

(or feline) Treg cells *in vitro* compared to Th cells (13, 45, 54). It is likely that HIV-1 LTR

has evolved to resist FoxP3-mediated mechanisms that inhibit *IL-2* expression in FoxP3+

Treg cells. Several recent reports have indicated that 1) Treg cells can serve as a

significant target population for HIV-1 infection in vivo and 2) these FoxP3+ Treg cells

support productive HIV-1 infection *in vivo*. Although about 5% of total CD4+ T cells from normal peripheral blood are CD25+FoxP3+ Treg cells, up to 50% of CD4+ T cells express FoxP3 in mucosal lymphoid organs from HIV-1 (46) or SIV (36) infected human or monkeys. In addition, it has been reported that virtually all human CD4+ T cells upregulate FoxP3 upon activation and have transient suppressive activity (55). Thus, FoxP3+ T cells can provide a significant number of target cells for HIV-1 infection. Remarkably, 13% of the FoxP3+ T cells are shown to be productively infected by SIV in the lymphoid organs of acutely infected animals (36) whereas 30-60% of memory CD4 T cells in these organs have been reported to harbor SIV proviral genomes (56-58). In comparison, <1% total infection are detected by qPCR of HIV-1 proviral genomes in blood CD4+ memory T cells from HIV-1-infected patients (59, 60). Therefore, FoxP3+ Treg cells are important target cells for HIV-1 infection and replication, at least in mucosal lymphoid tissues during acute infection.

The distinct modulation of LTR and *IL-2* expression by FoxP3 may be due to specific LTR NF-κB binding sequence, or to the unique FoxP3-mediated NF-κB activity and chromatin remodeling. Based on a recent report (61), the HIV-1 LTR-derived NF-κB site would have a 2-3 fold lower affinity relative to the MHC- or IL2-derived NF-κB sites (D. Holmes and L. Su, unpublished). FoxP3 may differentially affect the *IL-2* and HIV-1 promoter by altering the cooperative binding of p65-p50 with other transcription factors in T cells. While our data suggests that NF-κB is critical for FoxP3 enhancement of HIV-1 LTR, the role of NFAT should be clarified because NF-κB and NFAT share overlapping binding sequences at the LTR enhancer core. *IL-2* expression is critically

dependant on NFAT and AP-1 activity. Wu *et al* (37) describes a model in which FoxP3 binds NFAT to displace AP-1, thus inhibiting *IL-2* expression. The direct inhibition of NFAT transcriptional activity by FoxP3 (8, 39) may also lead to preferential inhibition of IL-2. Interestingly, FoxP3 binding at the promoters of *CD25* and *CTLA-4* is also accompanied by increased NFAT binding, suggesting that FoxP3 stabilizes NFAT on those promoters (37). While NFATc1 and NFATc2 have both been reported to enhance HIV-1 gene expression in some reports (41, 42), NFATc2 (or NFAT1) can also inhibit HIV-1 LTR by competitively binding to the NF-κB core sequences (43, 44). It is thus likely that FoxP3 may inhibit NFATc2 binding at competing LTR NF-κB sites and lead to increased NF-κB binding and LTR expression. However, our data do not support the involvement of NFAT. First, FoxP3 enhanced HIV-1 LTR expression in unstimulated Jurkat cells (Fig. 1A, 2A) where no NFAT activity is detected. Second, mutations in the LTR NF-κB binding sites blocked FoxP3-dependent enhancement, but mutations that specifically inhibit NFAT binding to the LTR (41) did not affect the enhancement (Fig. 2.4, and D. Holmes and L. Su, unpublished results). Finally, inhibition of NFAT activation by FK506 did not affect FoxP3-mediated enhancement of LTR (D. Holmes and L. Su, unpublished results), while enhancement of LTR by FoxP3 depended on NF-κB activation (Fig. 2.5A). Thus a new FoxP3 activity may be involved in modulating the NF-κB activity and in enhancing HIV-1 gene expression.

Similar to stabilized NFAT and FoxP3 binding at the promoters of *IL-2*, *CD25* and *CTLA-4* (37), the increase in NF-κB binding at the LTR in Jurkat cells may be due to a similar FoxP3-mediated stabilization. This is supported by the fact that IPEX mutations

in the forkhead DNA binding domain ablated FoxP3's activity on LTR (Fig. 2.3). However, it is not clear if FoxP3 directly binds to specific LTR sequences. The physical interaction of FoxP3 with NF-κB is also controversial. Although FoxP3 is shown to interact with NF-κB when overexpressed in 293T cells (39), others (37) and our study (D. Holmes and L. Su, unpublished results) have failed to detect such interaction. Similar to its interaction with NFAT (37), FoxP3 may also interact with NF-κB in a DNA sequence-dependent fashion.

A recent report has documented that FoxP3 represses HIV-1 LTR by inhibiting NF-κB in T cells as well as in 293 cells (40). In contrast, we observed that FoxP3 inhibited HIV-1 LTR in 293T cells but enhanced its activity in T cells (Fig. 2.5). The apparent discrepancy in T cells may be due to different experimental models and procedures. We analyzed HIV-1 gene expression mostly from T cells infected with reporter HIV-1 pseudotyped with VSV-G, whereas the previous report is based only on transient transfection of promoter-reporter assays. The FoxP3 effect on HIV-1 LTR also depends on the activation condition of T cells. We observed enhancement of LTR by FoxP3 in T cells either unstimulated or stimulated with anti-CD3/CD28. However, low or no significant enhancement by FoxP3 was observed following stimulation with PMA and ionomycin in T cells (D. Holmes and L. Su, unpublished results). The activation conditions used in the previous study are not clear (40). Therefore, transfection or infection methods, and relative levels of activation may contribute to the discrepancy. In addition, the C-terminal Forkhead domain of FoxP3 is required for the reported FoxP3 activity in 293T cells but not in T cells (40). Most human IPEX mutations reside in the

Forkhead domain, suggesting that the reported FoxP3 activity in inhibiting NF-κB and

HIV-1 LTR in T cells may not be a critical FoxP3 activity. Our data concludes that both

the N-terminus and Forkhead domains are essential for FoxP3 to enhance HIV-1 LTR,

consistent with the findings by Wu *et al.* (37) that both domains of FoxP3 are required

for repression of *IL-2* and activation of *CD25* and *CTLA-4*.

FoxP3 has recently been demonstrated to inhibit *IL-2* and *INF-γ* gene expression

by reducing the histone acetylation and thus altering their chromatin structure.

Conversely, promoters activated by FoxP3 (such as *CD25*, *GITR* and *CTLA-4*) have

increased levels of acetylation in FoxP3-expressing cells (38). Our findings support a

model wherein expression of FoxP3 leads to increased NF-κB occupancy at the HIV-1 LTR

and elevated histone acetylation in T cells. The altered chromatin structure at the LTR

may promote NF-κB occupancy in FoxP3-expressing T cells. Since the FoxP3-mediated

effect is correlated with increased AcH3 level at the LTR, *CD25* and *CTLA4* loci but

decreased AcH3 level at the *IL2* gene locus (Fig. 2.7A and (38)), FoxP3 may modulate

activity of specific HDAC or HAT in a promoter-dependent fashion in T cells.

Alternatively, recruitment of HDAC or HAT by NF-κB protein to HIV-1 LTR (62, 63) may

be altered by FoxP3 and contribute to the differential gene modulation.
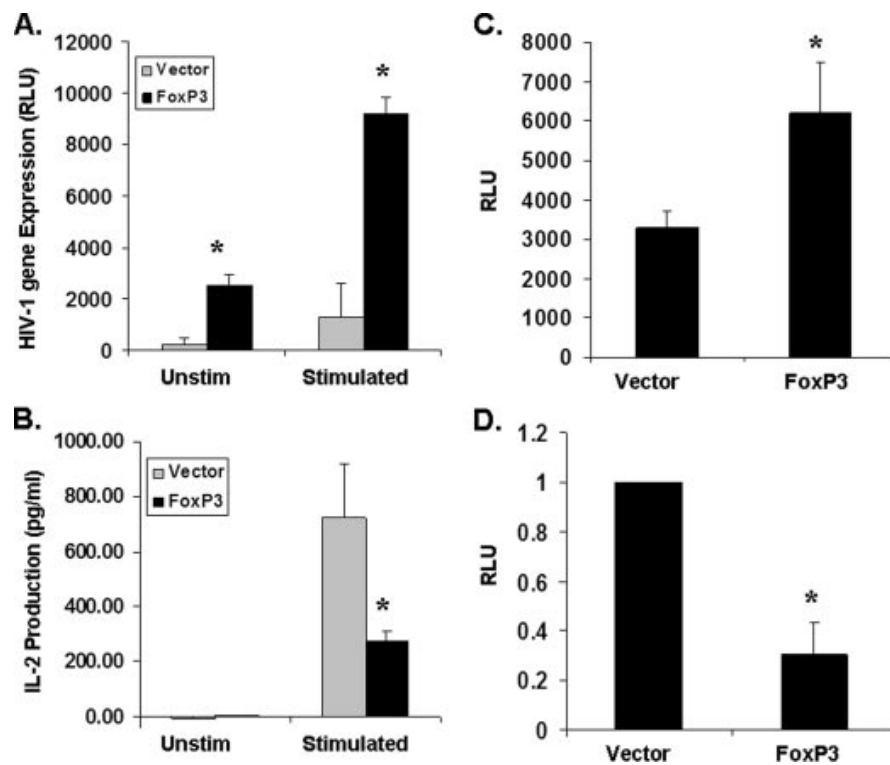
Acknowledgements

Figure 2.1. FoxP3 inhibits *IL-2*, but enhances HIV-1 gene expression in T cells.

(A) Vector or FoxP3 transduced Jurkat T cells were infected with VSV-G pseudotyped NL4-Luc virus and non-activated or CD3/CD28 stimulated. At 48 hours post-infection, cells were lysed and assayed for luciferase expression. **(B)** Vector control and FoxP3 transduced Jurkat cell were non-activated or activated with PMA and Ionomycin for 24 hours and supernatants assayed for IL-2 by ELISA. **(C)** Primary CD4+ T cells transduced with FoxP3 or control vector and infected with VSV-G/NL-4-Luciferase virus and relative HIV-1 gene expression was measured at 48 hpi. **(D)** Primary CD4+ T cells transfected with control vector or FoxP3 plasmid (sorted based on GFP) were activated with anti-CD3/CD28 and cultured for 2 days. Cells were harvested for relative IL-2 expression by real-time RT-PCR. Data represents 3 independent experiments done in triplicates. (*, p<0.05)
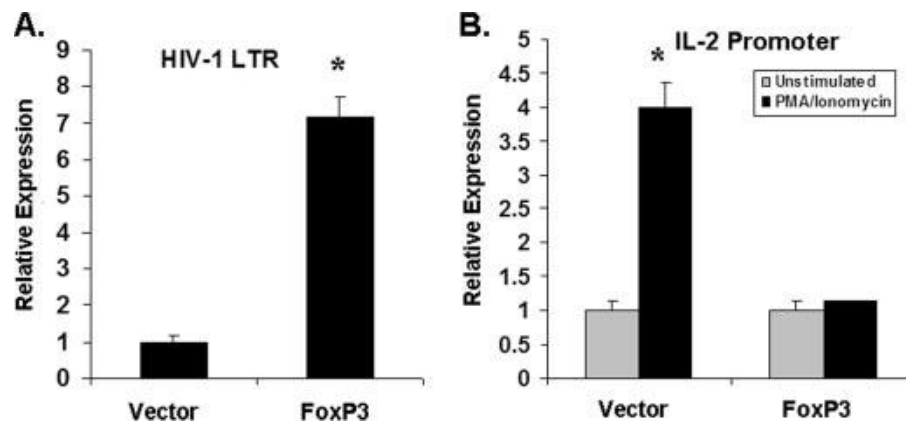
Figure 2.2. FoxP3 inhibits IL-2 promoter, but enhances HIV-1 LTR expression in T cells.

Jurkat T cells were transiently transfected with HIV-1 LTR **(A)** or IL-2 promoter **(B)** driven luciferase constructs in the presence of control vector or FoxP3 plasmid. For IL2-luciferase assay, Jurkat cell were non-activated or activated with PMA and Ionomycin at 36 hours post transfection. Luciferase gene expression relative to vector control cells was measured at 48 hpi. Data represents >5 independent experiments done in triplicates. (*, p<0.05)
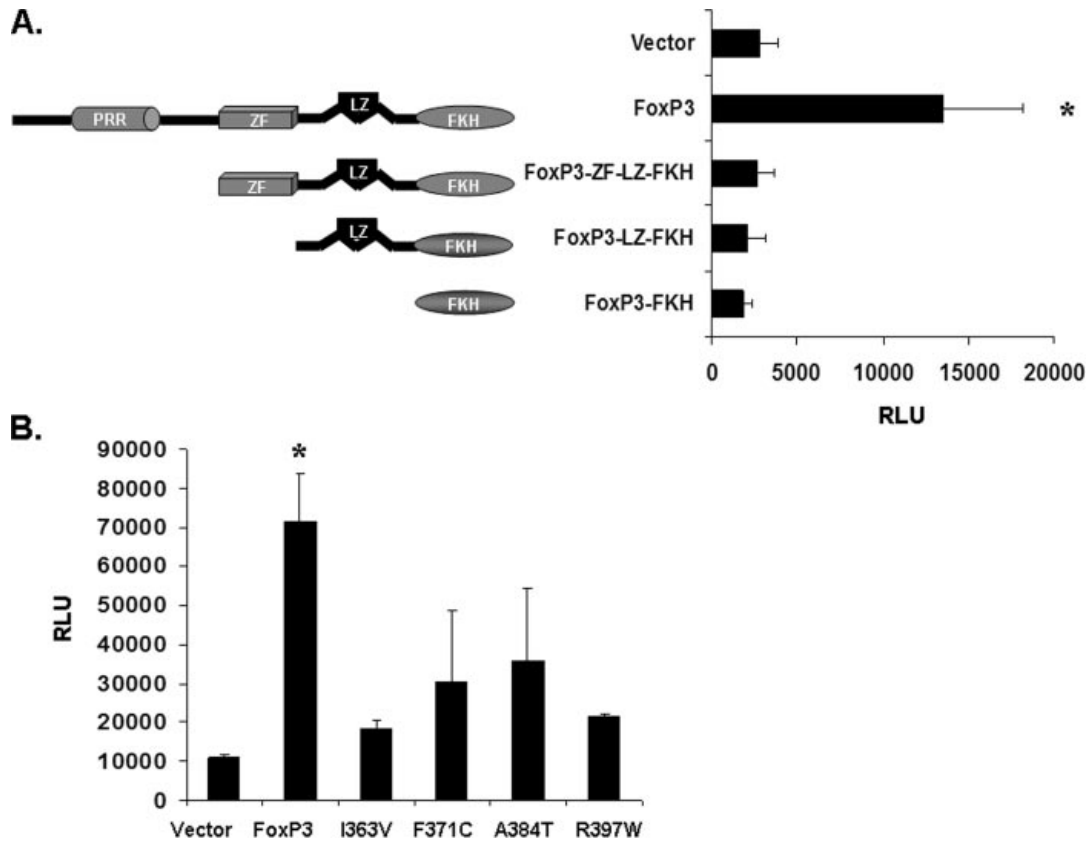
Figure 2.3. A critical activity of FoxP3 is required for LTR enhancement.

(**A**) Deletion mutants of FoxP3 were made as described in methods. Jurkat cells transduced with retrovirus containing no insert control (PG), wild type FoxP3 (FP3), FoxP3 C-terminus (FP3C, aa191-420), FoxP3 lacking the N-terminus and Zinc Finger (FP3LZFKH, aa241-420), or the Forkhead domain of FoxP3 (FP3-FKH, aa333-420) were infected with the VSV-G NL4-Luciferase virus and luciferase activity was measured 48 hours post infection. Proline-rich region (PRR), zinc finger (ZF), leucine zipper (LZ), and Forkhead domain (FKH) are labeled. (**B**) FoxP3 genes with indicated IPEX mis-sense mutations are cotransfected into Jurkat cells with HIV-1 LTR-luciferase. (*, p<0.05)
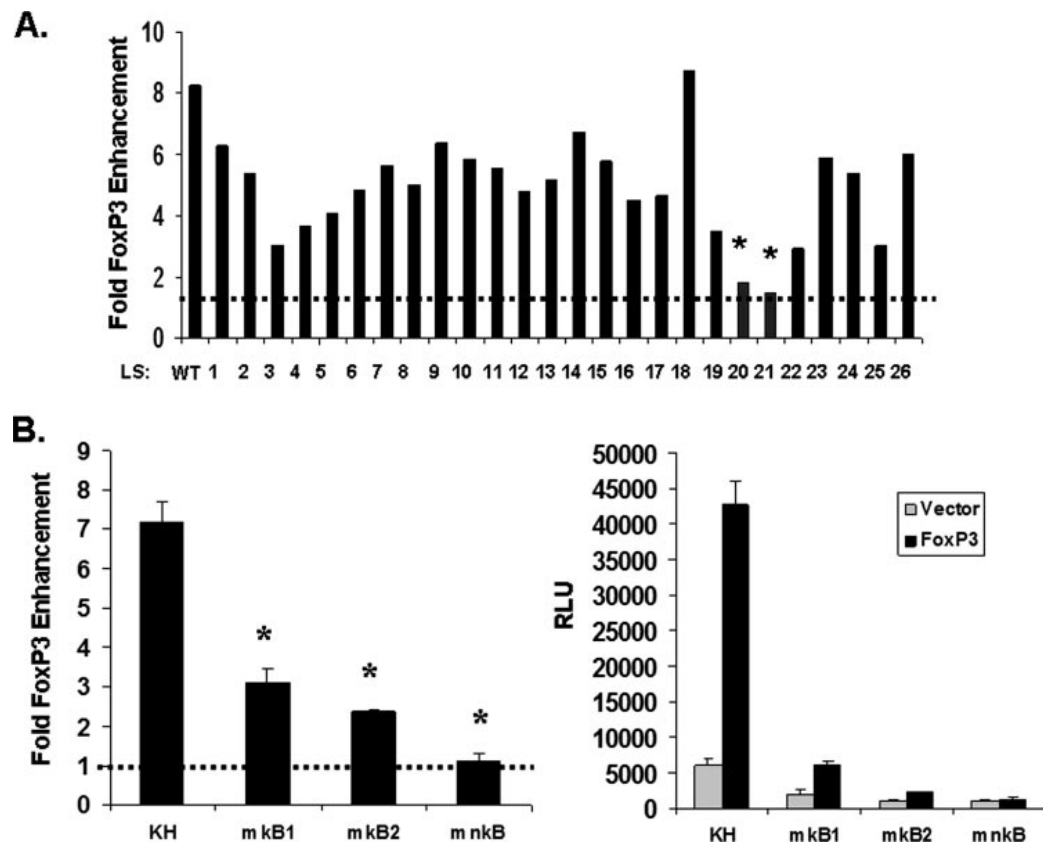
Figure 2.4 FoxP3-mediated LTR enhancement maps to the NFκB sites.

(**A**) Linker scanning (LS1-LS26) mutants (48) replacing every 18 bp of HIV-1 LTR were cotransfected into Jurkat cells with vector or FoxP3 as above and lysates were assayed for LTR-driven luciferase expression. Data are representative of 3 independent experiments each done in triplicates. LS20/LS21 mutants affect the two NFκB sites in the LTR. (**B**) HIV-1 LTR containing mutations ablating specific binding by NF-κB (41) were cotransfected with control vector or FoxP3 in Jurkat cells, and results are summarized from 3 independent experiments.
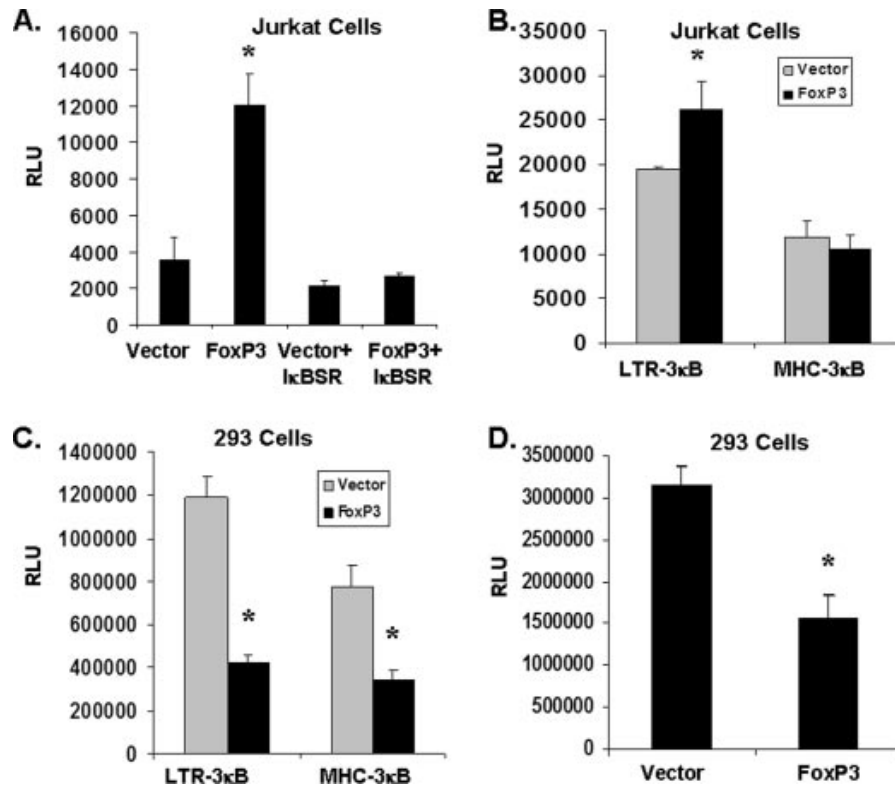
Figure 2.5 FoxP3 affects LTR expression via an NF-κB sequence-dependent and cell type dependent mechanism.

(**A**) LTR enhancement by FoxP3 is ablated by IκBα-super repressor (IκB-SR(50)). LTR-luciferase is transfected with IκB-SR and vector or FoxP3 plasmid into Jurkat cells. (**B/C**) LTR enhancement by FoxP3 is specific for the LTR NF-κB sites. Luciferase reporters driven by multiple copies of LTR or MHCII (50) NF-κB sites were transfected into Jurkat cells (B) or 293T cells (**C**) with vector control or FoxP3. (**D**) FoxP3 represses in 293T cells. 293T cells transfected with vector control or FoxP3 with HIV-1 LTR luciferase reporter plasmids. Experiments were done in triplicates and lysates are assayed for luciferase expression.

Figure 2.6. FoxP3 differentially affects the Histone 3 acetylation level of the *IL-2* and HIV-1 LTR promoters.
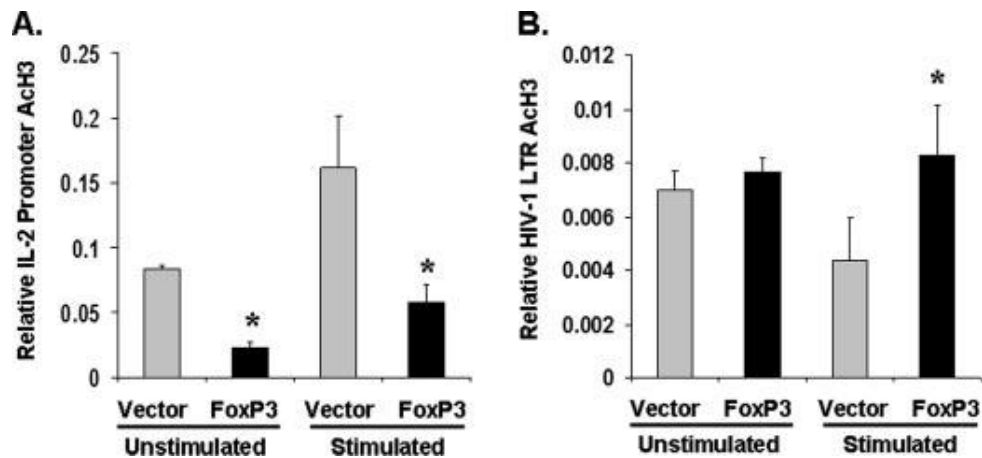
Jurkat A82 T cells with a copy of stably integrated HIV-1 LTR were transduced with vector or FoxP3 (>95% GFP+). Sonicated chromatins from activated Jurkat cells were IP'ed with control IgG or anti-acetyl-H3 Ab (AcH3). (**A**) FoxP3 reduces the acetylation of histone H3 at the *IL-2* promoter. ChIP analysis of vector or FoxP3 transduced Jurkat A82 T cells was performed. Samples were purified and subject to qPCR analysis with the IL2 promoter amplicon spanning the NFκB and NFAT/AP-1 sites (-226 to -133) in the human *IL-2* promoter. (**B**) FoxP3 increases H3 acetylation at integrated HIV-1 LTR locus.   The same Jurkat A82 T cells with a stably integrated HIV-1 LTR were analyzed by anti-AcH3 ChIP for HIV-1 LTR core enhancer sequences by qPCR. Error bars indicate SD of triplicate samples. *, $p < 0.05$
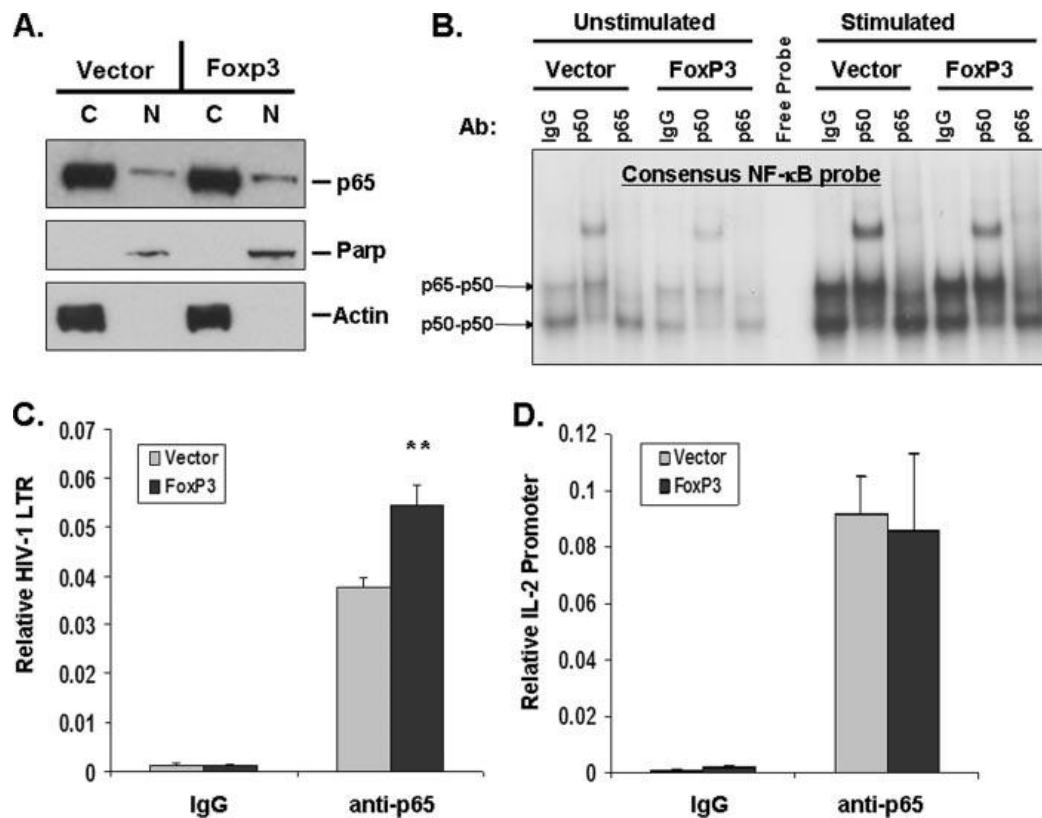
Figure 2.7. FoxP3 alters NF-κB occupancy to the LTR promoter.

 (**A**) FoxP3 expression does not affect NF-κB localization in the nucleus. Vector control and FoxP3 transduced Jurkat T cells were fractionated, cytoplasmic and nuclear lysates were separated by SDS-PAGE. Membrane was probed for NF-κB using anti-p65 antibody. (**B**) FoxP3 does not alter NFκB p65 and p50 binding to the MHCII-derived consensus NF-κB probe. Nuclear extract of transduced Jurkat T cells were left unstimulated or PMA/Ionomycin stimulated for 30 minutes and NF-κB binding was assessed. Antibodies specific for p65 and p50 were used for supershift. (**C-D**) Jurkat T cells with a stably integrated HIV-1 LTR were transduced with control or FoxP3-expressing vectors. Sonicated chromatins were IP'ed with control IgG or anti-p65 Ab. Samples were purified and subject to real-time qPCR analysis. Input chromatin (10% relative to each IP) was used to normalize samples. The relative p65 occupancy at the HIV-1 LTR (**C**) or IL-2 promoter (**D**) from a representative experiment is shown as relative anti-p65-specific ChIP signals divided by signals from control input chromatins. (******, p<0.01)
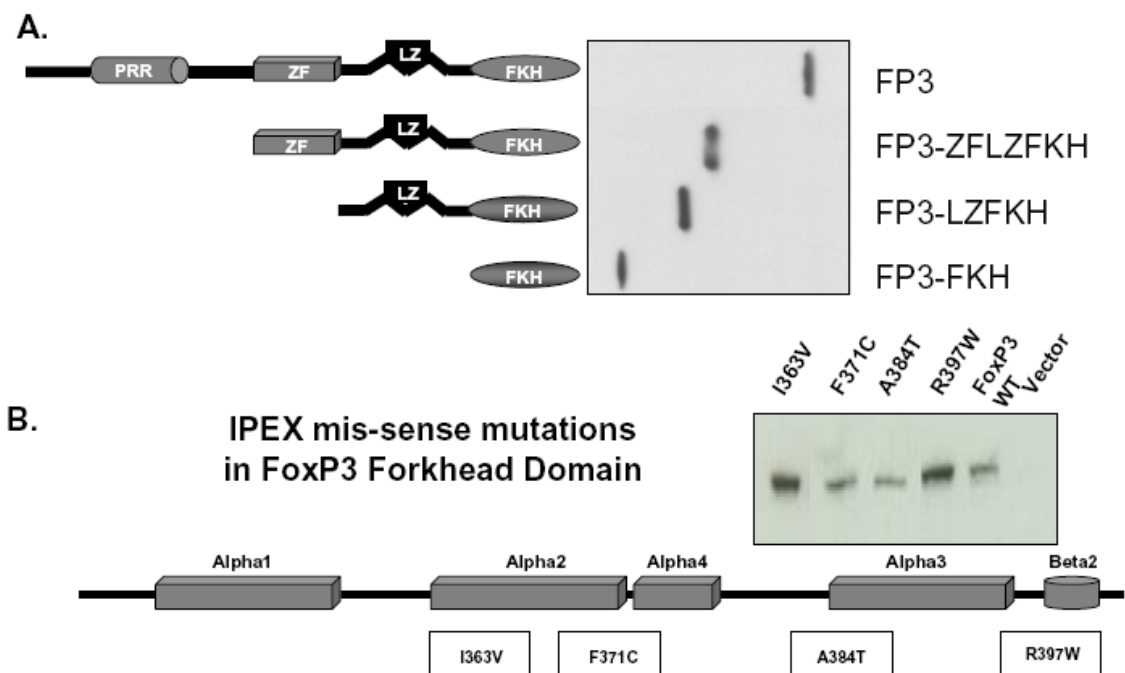
Figure S2.1

(A) Schematic diagram of FoxP3 protein domains and its deletion mutants, and their expression by Western blot of myc-tagged FoxP3 deletion mutants expressed in 293T cells. **(B)** Western blot of FoxP3 IPEX mutants in 293T cells and a schematic representation of mis-sense IPEX mutations relative to known forkhead domain structures.

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Chapter III

FoxP3 Inhibits HDAC1 Deacetylase Activity to Modulate Gene Expression in Human T cells.

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Abstract

FoxP3 plays a crucial role in the development and function of CD4+CD25+ Regulatory T cells. FoxP3 function in Tregs is characterized by the transcriptional regulation of several genes important for Treg function, including activating genes such as *CTLA4, GITR* and *CD25* while inhibiting *IL-2*, *IFN-γ* and *IL-4*. Previously, our group has shown that FoxP3 enhances the HIV-1 LTR through an NF-κB-dependent mechanism. Here, we describe a novel function for FoxP3 as an inhibitor of cellular deacetylase activity in Jurkat T cells and human Treg cells. FoxP3 inhibition of deacetylase activity is specific for the class I HDAC family member HDAC1. We show that FoxP3 associates with a large molecular weight complex in T cells. We also demonstrate that FoxP3 interacts with HDAC1, and the Proline-rich region is required for HDAC1 inhibition. Mutations in the forkhead domain that ablate FoxP3 function in Tregs also inhibit Foxp3/HDAC1 interaction and consequently ablates FoxP3 inhibition of HDAC1. Finally, we demonstrate that knockdown of HDAC1 in T cells inhibits FoxP3 regulation of promoter activity. Thus, FoxP3 modulates gene expression through the regulation of HDAC1 activity.

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Introduction

Regulatory T cells (Tregs) play a crucial role in immune function and homeostasis. Originally described as a subset of CD4+ cells expressing high levels of CD25, Tregs are able to control autoimmunity induced by neonatal thymectomy (1). The forkhead winged-helix family member FoxP3 is identified as the essential transcription factor necessary for Treg development and function (2-4). Originally described as an inhibitor of cytokines in T cells (5), several studies have demonstrated a gene signature of differentially regulated promoters associated with FoxP3 expression (6, 7). Initial studies delineating the molecular mechanisms of FoxP3, using the IL-2 promoter as a model promoter, have described multiple mechanisms of repression. FoxP3 interacts with the T cell-specific NFAT transcription factor to displace an activating NFAT/AP-1 complex at the *IL-2* enhancer (8). A FoxP3/Runx complex has also been shown to be necessary for complete FoxP3-mediated *IL-2* repression at a distal promoter region (9). More recently, FoxP3 has been shown to recruit a chromatin remodeling complex which includes a HAT/HDAC complex (10). Thus, FoxP3 is able to regulate gene expression by modulating factor binding and chromatin remodeling.

Histone deacetylases (HDAC) can be divided into 4 groups. Class I includes HDAC1-3 and HDAC8, and class II consists of HDAC4-7 and HDAC9, characterized by conserved sequence motifs in the catalytic HDAC domain (11). The remaining HDAC 11 (class IV) and sirtuin-family (class III) are functionally distinct based on their homology (HDAC11) to class I

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and II HDACs, and NAD$^+$ requirement (sirtuins), respectively. Histone acetylation is an integral component of gene transcription, and HDACs along with other chromatin remodeling factors are associated with T cell activation and Treg function. HDAC inhibitors (HDACi) have multiple effects on T cells, including repression of cytokine gene expression, cell cycle arrest and induction of apoptosis (12). Several groups have shown that TSA blocks *IL-2* gene expression along with other important inflammatory cytokine (12, 13). The role of HDACs in Treg function is even more revealing. *In vivo* TSA administration in mice increases Treg numbers and enhances their intrinsic suppressive activity (14). Promoters regulated by FoxP3 binding have an enhanced or reduced level of histone acetylation associated with increased or reduced gene expression, respectively (15, 16). Furthermore, Li *et al* has described a HAT/HDAC complex, Tip60/HDAC7, that is recruited by FoxP3 to the *IL-2* promoter, and its disruption inhibits FoxP3-mediated *IL-2* repression.

The repressive effect on gene expression by class II enzymes has been well documented and extensively reviewed (recently by Martin *et al*. (17)). Class IIa enzymes (HDAC4,5,6,7 and 9) can regulate transcription by direct and indirect mechanisms, but multiple studies have questioned the role of the N-terminal deacetylase domain in repression. The deacetylase activity of class IIa enzymes is postulated to originate from associated class I HDACs. In support of this hypothesis, Lahm *et al.* determined that the intrinsic deacetylase activity of class IIa enzymes is 1,000-times less efficient due to a conserved histidine residue in the catalytic domain. Replacement of this residue by tyrosine, conserved in class I enzymes, restored class IIa deacetylase function to class I levels (18).

In this study we report that FoxP3 regulates promoters similar to HDACi. We show

expression of FoxP3 inhibits HDAC activity in Jurkat T cells and primary Tregs. Importantly,

we describe a novel role of FoxP3 as an inhibitor of HDAC1-specific deacetylase activity,

which is important in FoxP3 regulation of gene expression. This HDAC-dependent inhibitory

function of FoxP3 requires the N-terminal regulatory domain, and is also inhibited by

forkhead mutations that block FoxP3 function. Finally, knockdown of HDAC1 alters FoxP3

regulation of *IL-2* and HIV-1 LTR activity. This new activity of FoxP3 gives us insight into the

complex mechanisms of FoxP3-mediated gene regulation.

Results

FoxP3 modulates gene expression via HDAC-related mechanisms. It is well established that FoxP3 can repress the *IL-2* promoter, and previous work from our lab demonstrated FoxP3 enhances HIV-1 gene expression (16). Interestingly, HDAC inhibitors also enhance HIV LTR expression and inhibit *IL-2*. We therefore wanted to determine the mechanism of FoxP3 repression or activation of targeted promoters in the presence of HDACi. Jurkat cells transduced with control vector or FoxP3 (>90% efficiency by GFP expression) were infected with VSV-g pseudotyped HIV-1 NL4-luciferase virus. Infected cells were either unstimulated or activated with plate bound CD3 and CD28. Treatment of these cells 24 hours post infection with the HDAC inhibitor TSA resulted in a 6 fold increase in HIV-1 gene expression in unstimulated or CD3/CD28 stimulated control cells (Fig 3.1A). FoxP3 expressing cells had enhanced LTR expression compared to control cells with or without activation, but TSA addition was unable to enhance LTR expression. We next determined the effect of HDACi on FoxP3 repression of *IL-2*. Jurkat T cells were transfected with IL-2 luciferase expressing plasmid in the presence or absence of TSA. FoxP3 inhibited the IL-2 promoter by 2-3 fold, and TSA similarly inhibited IL-2 promoter. Addition of both TSA and FoxP3 increased additively repression of IL-2 (Fig. 3.1B). To further this finding to the endogenous *IL-2* promoter, Jurkat cells were transduced with vector or FoxP3 retrovirus and stimulated with PMA and Ionomycin for 16 hours in the presence or absence of TSA. TSA inhibited *IL-2* expression 4 fold, to similar levels seen with Foxp3 expression, and FoxP3 in the presence of TSA repressed *IL-2* (Fig. 3.1B). Therefore the activation of LTR or inhibition

of *IL-2* by FoxP3 and TSA was through the same pathway and we proposed that FoxP3 might act like an HDACi.

Reduced class I HDAC activity in human T cells expressing FoxP3. Since FoxP3 works similar to HDACi in human T cells, we next wanted to determine the effect of FoxP3 on global HDAC deacetylase activity. Utilizing a live cell permeable HDAC substrate, we were able to determine the intracellular HDAC activity of cells in culture. FoxP3 expressed by retroviral transduction in Jurkat cells decreased global HDAC activity, approximately 30-50% reduction as assessed by HDAC assay (Fig. 3.2B). Addition of TSA completely reduced HDAC activity to equivalent background levels. Similarly with nuclear lysates, HDAC activity was decreased in Foxp3 transduced Jurkat cells compared to vector control when normalized to total protein (Fig. S3.1). We next wanted to determine if HDAC activity is lower in primary Treg cells. Human CD4+ T cells purified from whole blood were sorted into CD4+CD25- (Teff cells) and CD4+CD25+ (Treg cells) populations and activated by plate-bound CD3 and CD28 antibody stimulation. The relative HDAC activity in Tregs cells was reduced by ~25% compared to CD4+CD25- population (Fig. 3.2B). FoxP3 is a member of the Foxp subfamily of Forkhead family transcriptional regulators. In order to determine if the HDAC inhibitory function of FoxP3 was also found in other family members, FoxP2 and FoxP4 were expressed in Jurkat T cells by retroviral transduction. When total HDAC activity was assayed, FoxP2 and FoxP4 also reduced global HDAC levels by 40% and 25%,

respectively (Fig. 3.3). Thus, other Forkhead P family members also have HDAC inhibition

activity.

FoxP3 inhibits HDAC1 activity. We next wanted to determine the specific HDAC

inhibited by FoxP3. Since class I HDACs are responsible for most , if not all, of the

intracellular HDAC enzymatic activity (18), we overexpressed FoxP3 and various class I

(HDAC1, 2 and 3) and class II HDACs (HDAC4) in 293T cells. Flag-tagged HDAC1, 2, 3 and

HDAC4 were immunoprecipitated from equal amounts of nuclear lysates in the presence or

absence of FoxP3. HDAC deacetylase activity in the precipitated complex was assessed

following addition of an acetylated substrate specific for HDACs. FoxP3 was able to

efficiently inhibit HDAC 1 but not HDAC 2-4 (Fig. 3.4A). To ensure the difference was not

due to HDAC expression or immunoprecipitation, the IP'ed HDAC1-4 proteins were

measured by western blot analysis and both HDAC and FoxP3 expression levels were similar

between transfections (Fig. 3.4B inset). To ensure that inhibition of HDAC1 occurred in a

more physiologically relevant context, FoxP3-dependent HDAC inhibition was assayed in

Jurkat cells over-expressing HDAC1 by retroviral transduction. HDAC1 expression increased

the cellular HDAC activity above vector control, and FoxP3 was also able to inhibit HDAC1-

specific deacetylase activity (Fig. 3.4B). As a control, we show that nuclear levels of HDAC1

in Jurkat cells were not affected by FoxP3 expression (Fig. 3.4C). To define the functional

domain involved in the HDAC inhibition activity of FoxP3, a number of FoxP3 mutants with

deletions and point mutations were tested. Flag-tagged HDAC1 immunoprecipitated with

FoxP3 lacking the N-terminal proline-rich regulatory domain (mychFP3-C) had impaired

HDAC1 inhibition, thus the forkhead, leucine zipper and zinc finger domains are not

sufficient to inhibit HDAC1 deacetylase activity (Fig. 3.4D). Interestingly, the WWRR FoxP3

mutant, which is unable to repress *IL-2* or reprogram Treg cells (8), also has impaired HDAC1

inhibition activity when over-expressed in 293T cells (Fig. 3.4D). As a control, the FoxP3

mutant WRR which has slightly reduced function *in vivo* compared to wild type FoxP3, also

inhibited HDAC activity, but slightly lower than wild type FoxP3 (Fig. 3.4D). Similarly, when

the WWRR mutant was over-expressed in Jurkat T cells, it had drastically reduced HDAC-

inhibitory function compared to wild type FoxP3 (Fig. 3.4E). Therefore, as in *IL-2* repression

and Treg reprogramming, both the N-terminal regulatory region and a functional forkhead

domain of FoxP3 are required for HDAC1 inhibition by FoxP3.

There are several mechanisms of HDAC regulation, including gene expression,

posttranslational modification and association with a multi-subunit protein complex. We

measured co-segregation of HDAC1 and FoxP3 by size-fractionation. As reported previously

by Li *et al.*, FoxP3 fractionates into high and low molecular weight complexes (19, 20).

Similarly, we see FoxP3 fractionate into high molecular weight fractions (Fig. S3.2). HDAC1

is found in similar weight fractions as FoxP3, along with other chromatin remodeling factors

(data not shown) consistent with Li *et al.* We next wanted to determine if FoxP3 interacts

with HDAC1 to regulate its deacetylase activity. 293T cells transfected with myc-tagged

FoxP3 with or without flag-tagged HDAC1 were immunoprecipitated with Flag M2 antibody.

FoxP3 pulldown was enriched when HDAC1 and FoxP3 were coexpressed (Fig. 3.5A, left

panel). In Jurkat T cells transduced with myc-FoxP3, endogenous HDAC1 was also co-IP'ed

with FoxP3 (Fig. 3.5A, right panel). In order to map the domain of FoxP3 required for HDAC1 interaction, 293T cells were transfected with myc-FoxP3 or successive N-terminal truncation mutants of FoxP3 deleting the proline-rich region (FoxP3-C), the zinc-finger (FoxP3-LZ-FKH), and all domains up to the forkhead (FoxP3-FKH). HDAC1 associated with FoxP3 through the forkhead domain and importantly, the FoxP3 mutant WWRR did not bind HDAC1 (Fig. 3.5B). The fact that the WWRR mutant is unable to associate with HDAC1 and is unable to inhibit HDAC1 activity suggests that the interaction of HDAC1 with FoxP3 is important for FoxP3-mediated HDAC inhibition.

HDAC1 is required for FoxP3 inhibition of *IL-2*. In order to translate FoxP3 inhibition of HDAC1 to gene regulation, we utilized validated siRNA from Dharmacon specific to HDAC1 in IL-2 luciferase assays. Jurkat cells were transiently transfected with a scrambled siRNA or HDAC1 siRNA in the presence or absence of FoxP3. In accordance of TSA inhibition of *IL-2*, IL-2-driven luciferase levels were reduced when HDAC1 was knocked down, suggesting that HDAC1 is an important target in HDACi-mediated IL-2 suppression (Fig. 3.6A). Interestingly, reduced HDAC1 expression in T cells also decreased the relative inhibition of *IL-2* expression by FoxP3 (from 4.5x to 2.5x), while FoxP3 repression was unaffected by HDAC1 knockdown (Fig. 3.6A and data not shown). To ensure that HDAC1 inhibition was also occurring at the endogenous *IL-2* promoter, Jurkat T cells stably expressing HDAC1 shRNA from a lentivirus were activated with PMA and ionomycin, and IL-2 secreted into the media was measured by ELISA. Under conditions where HDAC1 levels

were knockdown ~40% by protein level determination, expression of *IL-2* was inhibited by

40% compared to a nonspecific shRNA control cells, similar to the effect seen in transient

transfection assays (Fig. 3.5B). Under the same condition, FoxP3-specific activity was also

reduced by ~40% compared to non-specific shRNA control (Fig. 3.6B). We next determined

the role of HDAC1 in the regulation of FoxP3-mediated activation of HIV-1 LTR. HDAC1

knockdown reduced FoxP3 activity at the LTR by approximately 2 fold (Fig. 3.6C). Thus,

FoxP3-mediated regulation of the LTR and *IL-2* promoters goes through an HDAC1-specific

activity.

Discussion

We have previously determined that FoxP3 acts as a transcriptional activator of HIV-1 while suppressing the *IL-2* promoter in human T cells. We report that FoxP3 and HDAC inhibitors function through similar pathways to modulate gene expression, and we found that FoxP3 inhibited HDAC activity in human Treg cells. Ectopic expression of FoxP3 in Jurkat T cells resulted in decreased intracellular HDAC activity, and CD4+CD25+ Tregs isolated from human PBMC's also had lower levels of HDAC activity compared to CD4+CD25- T cells. We show that HDAC1 is the main target for inhibition by FoxP3, and that both the N-terminal proline-rich region and the forkhead domain required for FoxP3 activity *in vivo* were also required for HDAC1 inhibition. We further demonstrate that FoxP3 exists in high molecular weight complexes and co-fractionates with HDAC1. FoxP3 interacts with HDAC1 and this association requires the Forkhead-domain. To give this activity biological relevance, we show that the Forkhead mutant (Foxp3-WWRR) lacking FoxP3 function was unable to bind to and inhibit HDAC1. Finally, we report a previous unknown function for HDAC1 in *IL-2* activation. shRNA knockdown of HDAC1 inhibited endogenous *IL-2* expression and reduced the relative inhibition of *IL-2* by FoxP3. Thus, FoxP3 acts like an HDAC inhibitor to modulate gene expression in T cells via inhibition of HDAC1 activity.

HDAC inhibitors (HDACi) have been extensively studied as anticancer agents. Several studies have demonstrated the effect of HDACi on cell cycle progression and apoptosis of tumor cells, but the role of HDACi *in vivo* may be broader yet. Administration

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of HDACi reduces the severity of graft-versus-host disease in mice (21), while HDACi reduces

the *in vivo* levels of proinflammatory cytokines *INF-γ*, *TNF-α* and *IL-1-β* (22), which are

implicated in GVHD disease progression (23). Still others have shown that addition of the

HDACi TSA results in a decrease in *IL-2* gene expression in T cells (12, 13), although the

specific HDAC targeted in these studies was not identified. Here, we demonstrate that

HDAC1 is required for *IL-2* transcription and is the likely target of TSA in these studies.

HDACs play a central role in chromatin remodeling and transcriptional regulation.

Recently, Tao *et al.* implicated Tregs as an immunologically relevant target of *in vivo* HDACi

activity. SAHA directly affected Foxp3+ Treg cells by increasing the *in vivo* cellularity and,

more intriguingly, enhancing Treg suppressive function. The mechanism of enhanced

suppressive activity is unknown, although HDACi increased the levels of Foxp3 and the cell

surface expression of Treg-associated inhibitory molecules such as GITR and CTLA-4 (14).

Also, HDACi was shown to enhance the acetylation of FoxP3, and mutation of lysine

residues abrogated FoxP3 function. Although we were unable to demonstrate FoxP3

acetylation (data not shown) it would be interesting to determine if FoxP3 inhibition of

HDAC1, or HDAC1 knockdown itself, can lead to enhanced FoxP3 acetylation and activity.

To date the only known HDACs associated with FoxP3 function are HDAC7 and

HDAC9. Li *et al.* described a mechanism wherein a HAT/ HDAC complex including

Foxp3/Tip60/ HDAC7 is required for *IL-2* repression (10). HDAC9-/- mice have Treg cells that

are more suppressive, pointing towards a role of class II HDACs in Treg function (14). Unlike

our results, Hancock *et al.* demonstrated that total levels of HDAC activity in Treg cells was

increased compared to CD4+25- cells, explaining that HDAC9 transcript levels were increased while expression of all other class I and class II HDACs were unchanged. One explanation for this discrepancy could be murine versus human T cells used in the two studies.   It is also unclear at what point following *in vitro* activation the HDAC levels were assayed. Interestingly, a recent paper showed that intrinsic HDAC deacetylase activity between class I (HDAC 1, 2, 3 and 8) and class IIa (HDAC4,5,7 and 9) differed by 1,000 fold, pointing to a single amino acid difference in the HDAC catalytic domain between these two classes (18). Thus, most of the deacetylase activity of class II HDACs can be attributed to associated class I HDACs. With this in mind, it will be important to determine the possible role of class II enzymes in association with class I enzymes in FoxP3-mediated gene regulation.

How FoxP3 inhibits HDAC1 activity is unclear. Only a few reports have been published on viral proteins targeting HDACs. Adenovirus early proteins bind to and inhibit several HDACs following infection (24-26). HDACs are regulated by posttranslational modification, association with multisubunit complexes, and cofactor availability (27). Unlike Class IIa HDACs, which are regulated by shuttling between the cytoplasm and the nucleus, class I HDACs are predominantly nuclear. Studies have shown that HDAC1 is constitutively phosphorylated *in vivo*, although mutation of specific phosphorylated serines had little consequence on intrinsic deacetylase activity *in vitro*. Since the protein levels of HDAC1 are unchanged upon FoxP3 expression (Fig. 3.4C), transcriptional regulation of HDAC1 by Foxp3 can be ruled out. FoxP3 is associated with a large molecular weight complex that includes Brg-1 and MBD3, components of the SWI/SNF and Mi2/NuRD complex respectively (19, 20).

The Mi-2/NuRD complex contains HDAC1 and HDAC2, and Foxp3 is found in a similar high molecular weight complex as HDAC1 (Fig. S3.2). FoxP3 did not seem to alter HDAC1 association with these multi-subunit complexes (unpublished results). Interestingly, HDAC1 itself can be regulated by acetylation. A HAT/HDAC complex containing p300 and HDAC1 occurs in the nucleus, and HDAC1 is acetylated by p300 resulting in decreased HDAC1 enzymatic activity (28). FoxP3 has previously been shown to associated with p300 by immunoprecipitation (29), and the ability of FoxP3 to regulate HDAC1 by acetylation or other modification will be of future interest.

The downstream target of this reduced HDAC1 activity in Treg cells is unclear. FoxP3 binding to and regulation of targeted promoters is associated with changes in histone acetylation (15, 16). A simple model wherein FoxP3 recruits HAT or HDAC complexes to enhance or inhibit acetylation and promoter activity would explain this finding. FoxP3 inhibition of HDAC1 is likely one of many mechanisms of FoxP3-mediated gene regulation. For instance, although TSA inhibits *IL-2*, *CD25* expression levels is relatively unchanged by TSA in non-Treg cells (12). HDACs are also known to regulate gene expression through deacetylation of non-histone proteins (30, 31). Previous reports have shown that HDACi repression of *IL-2* is associated with inhibition of NF-κB activation in Jurkat cells (13). FoxP3 inhibition of HDAC1 could lead to reduced NF-κB activity and thus reduced *IL-2* expression. Conversely, our previous data demonstrates that Foxp3 enhances HIV-1 LTR through an NF-κB-dependent mechanism (16). TSA enhances HIV-1 LTR by both histone and non-histone acetylation-dependent mechanisms. TSA has been shown to relieve LTR repression by acetylation of histones and "relaxing" the chromatin to enhance gene expression (32-34).

Alternatively, TSA activation of the LTR requires the NF-κB sequences, pointing toward transcription factor-dependent enhancement. FoxP3 may inhibit HDAC1 to modulate the activity of NF-κB in T cells. Several groups have shown that HDACi can both activate (35, 36) and repress (13, 37-39) NF-κB activity depending on the cell type and the promoter environment. Therefore, FoxP3 inhibition of HDAC1 might play a role in regulation of transcription factor activity, as well as in histone acetylation and chromatin remodeling.

Materials and Methods

      Plasmids, antibodies and cells. FoxP3 was cloned into the HSPG retrovirus vector as previously described (40). FoxP3 deletion mutants were described previously (16). FoxP3 mutants WRR and WWRR were a obtained from Ajana Rao and described previously (8). Human Foxp2 and Foxp4 were obtained from (   ), myc-tagged and subsequently cloned into HSPG vector. Jurkat cells were maintained in RPMI 1640 (Gibco BRL), 293T cells were maintained in DMEM and primary human T cells were maintained in Iscoves MEM, supplemented with 10% FBS (Sigma), 2mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml Streptomycin. Primary cells are maintained in 10% FBS supplemented Iscoves MEM (Gibco-BRL). CD4$^+$ primary T cells were purified from Ficoll separated PBMC by negative selection (>95% purity) (MACS human CD4+ T cell isolation kit) and CD4+CD25+ Regulatory T cells were purified by MACS human Regulatory T cells isolation kit (Miltenyi-Biotech, Auburn, CA). T reg cell purity was determined by anti-human CD25-PE (Miltenyi) and CD4-FITC (BD Biosciences) staining and dead cells were gated out by 7AAD (Molecular Probes). CD4+CD25+ cell purity was > 95%. T cells were activated by anti-CD3/CD28 mAb crosslinked by plate-bound anti-mouse IgG as previously described (41). Antibodies used in Western Blot include Flag M2 (Sigma), myc monoclonal 9E10 and HDAC1 polyclonal rabbit antibody (Santa Cruz).

Retrovirus production and transduction. 293T cells were co-transfected with retroviral vector, VSV-G and gag/pol containing plasmids as previously described (40). For production of single cycle HIV-Luc reporter virus, 293T cells were transfected with VSV-G and HIV-Luc plasmids by Effectene (Qiagen, Valencia, CA). For retroviral transduction, Jurkat cells were spin-inoculated with HSPG vector control or FoxP3 retrovirus to >95% efficiency as measured by GFP expression.

Transfection of Jurkat T cells and luciferase assay. Jurkat cells were transfected by electroporation. Briefly, 1×10^6 cells were incubated with 15ug empty vector or Foxp3 and 1ug IL-2 luciferase plasmid along with 500ng of a control GFP plasmid. 24 hours post transfection, Jurkat cells were activated with PMA (25ng/ml) and Ionomycin (1um) for 24 hours. Cells were lysed in 1x reporter lysis buffer (promega) and luciferase expression was determined by Luciferase Assay System (Promega). Luciferase readings were normalized to cell number and % GFP expression for every sample by Guava. Experiments were done in triplicates and repeated at least three times.

293T cell transfection, Immunoprecipitation and Western Blot. 293T cells were transfected using Effectene transfection reagent (Qiagen) per manufacturer recommendations. Cells were fractionated into cytoplasmic and nuclear lysates and quantitated by BCA protein assay kit (Pierce). 500ug nuclear lysates were incubated overnight with 2ug Flag antibody (Sigma) followed by Protein A bead incubation. For HDAC

assay, beads containing immunoprecipitated complex were washed 4X with PBS and HDAC

activity was analyzed by Flour De Lys HDAC assay as described below. For IP-western, beads

were washed in modified RIPA buffer and subject to SDS-page followed by transfer to PVDF

membrane and visualization by Western Blot.   FoxP3 was probed with an anti-FoxP3

antiserum (42) followed by secondary α‑rabbit‑IgG‑HRP (1:10,000) (Amersham

Biosciences) and visualized by ECL (Amersham Biosciences).

Gel Filtration Chromatography. Nuclear lysates isolated from 293T cells were

filtered through Ultrafree-MC 0.22μm columns prior to chromatography. 0.5 mg of total

protein was run on a Superose 6 10/300GL (Amersham Bioscience) gel filtration column

using a Duoflow chromatography system (Bio-Rad) in PBS with a flowrate of 0.3ml/min.

Fractions were collected in 2ml aliquots and subjected to analysis by Western blot.

HDAC Flourimetric Cellular Activity Assay. $1x10^5$ Jurkat E6.1 cells were incubated

with Flour de lys intracellular HDAC substrate (Biomol, Plymouth Meeting, PA) for 1 hour at

37^oC. Cells were lysed per manufacturer's protocol and Fluorescence was measured at

excitation 350 and emission 460 on a Fluostar plate reader. For HDAC activity of

immunoprecipitated complex, 20ul agarose beads are incubate with HDAC substrate in

HDAC assay buffer for 30 minutes at 37^oC and developed per manufacturer

recommendation. Equal bead/Immunoprecipitated HDAC complex is visualized by Western

Blot. For primary cell HDAC assay, cells stimulated for 3 days as described above were

assayed under similar conditions as Jurkat T cells. Experiments were done in triplicate and

are representative of at least 2 independent experiments.

IL-2 measurement by ELISA. Jurkat T cells transduced as above were cultured at

$5x10^5$ cells/ml and stimulated with PMA (25ng/ml) and Ionomycin (1uM) for 18 hours.

Supernatants were collected and secreted IL-2 was quantitated by the Human IL-2 ELISA kit

per manufacture's protocol (BD Biosciences, San Diego, CA). Experiments were done in

triplicate with 2-3 independent experiments.

Statistical analysis. For statistical analysis, a two-tailed Student *t*-test was employed

where P<0.05 was considered significant.
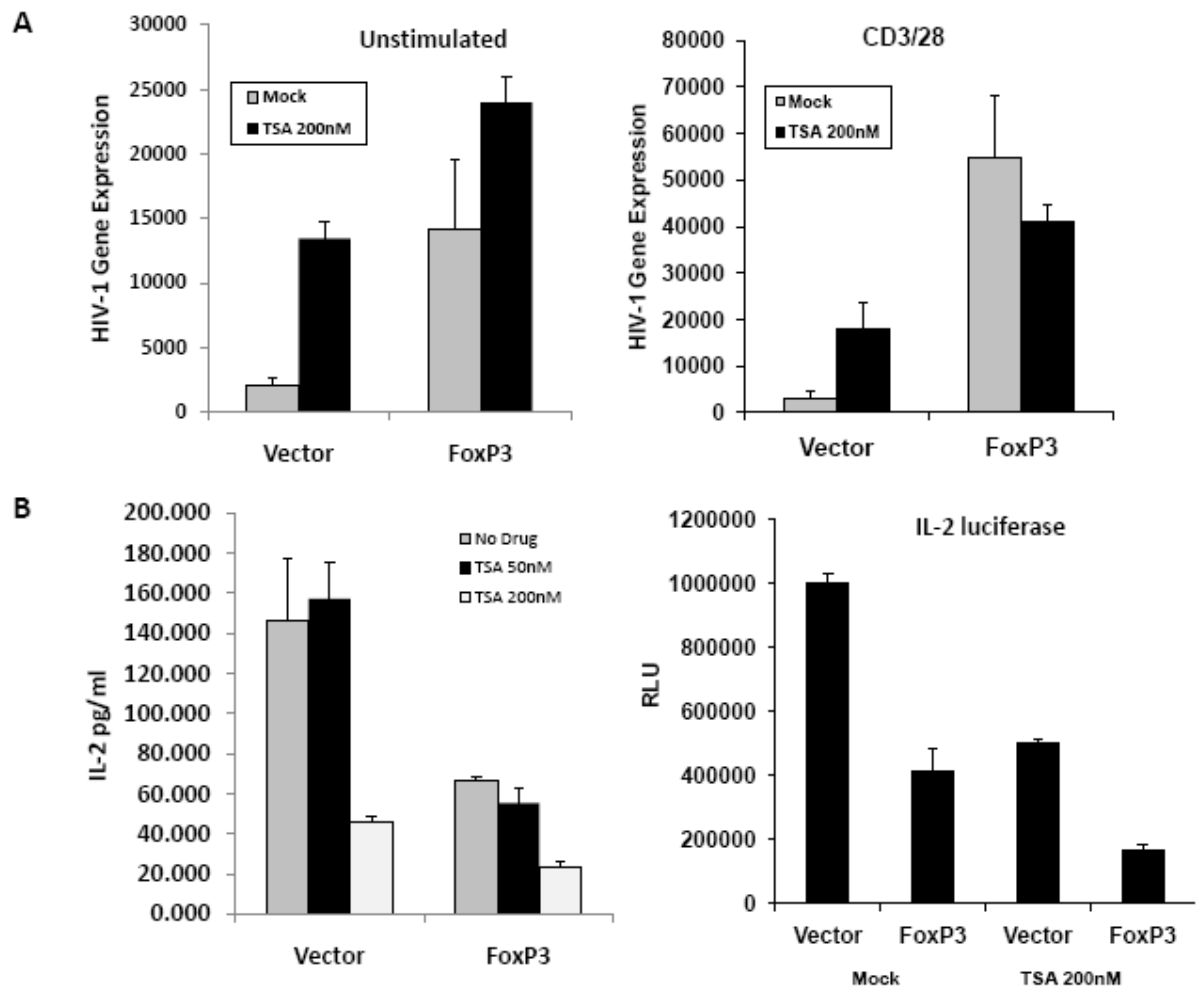
Acknowledgements

Figure 3.1. FoxP3 modulates gene expression similar to TSA.

(A) HIV-1 activation by FoxP3 is insensitive to TSA. Jurkat cells transduced with empty vector or FoxP3 are infected with VSV-g pseudotyped HIV-1 NL4-luciferase reporter virus. 24 hours post infection, unstimulated or CD3/CD28 stimulated cells are treated with mock or 200nM TSA for 24 hours. **(B)** FoxP3 inhibits IL-2 similar to TSA. Jurkat cells are transfected with empty vector or FoxP3 and treated with 200uM TSA for 24 hours. Cells are lysed and luciferase is determined. Data represents multiple experiments done in triplicate.
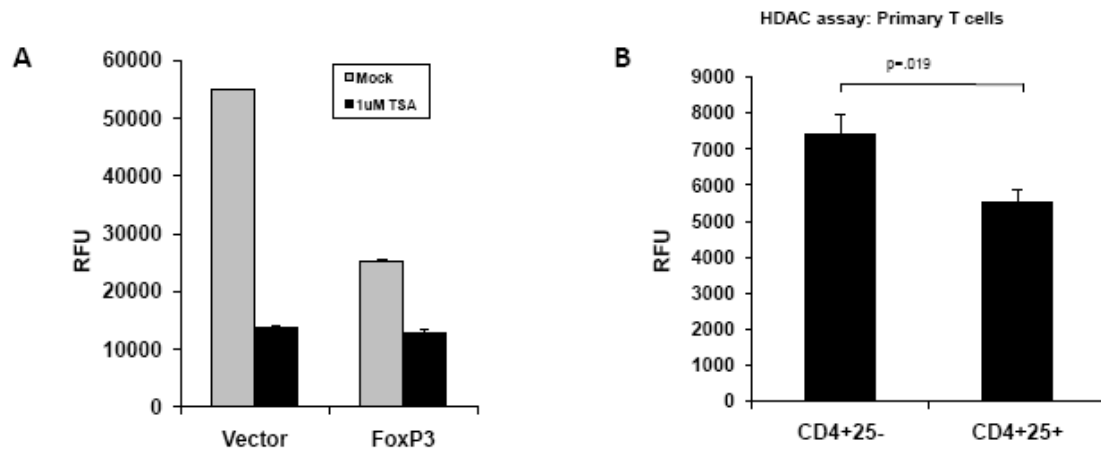
Figure 3.2. FoxP3 inhibits global HDAC activity in human T cells.

(A) Primary human effector T cells (CD4+ CD25-) and Treg cells (CD4+ CD25+) sorted from PBMC's are activated by plate bound CD3/CD28 . Live cells are cultured with HDAC substrate for 2 hours . Total cellular HDAC activity is represented at % HDAC inhibition compared to CD4+ CD25- cells. **(B)** Jurkat cells transduced with vector or FoxP3 were cultured with cell permeable HDAC substrate. Cells were lysed and HDAC activity was assessed by fluorometric analysis with the Flour De Lys Assay as described in material and methods. Data are representative of multiple experiments done in triplicate.
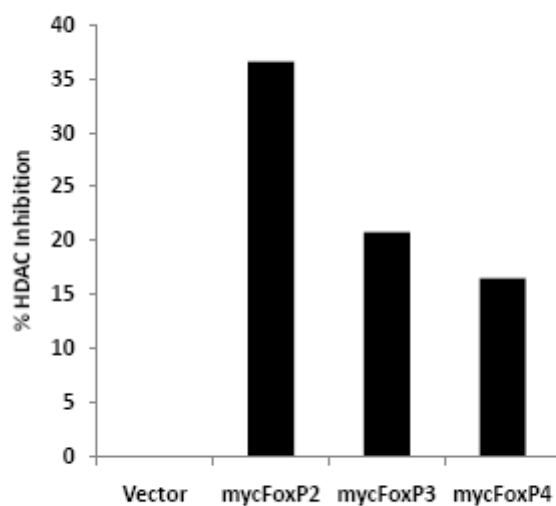
Figure 3.3. Other forkhead family members inhibit HDAC activity.

FoxP2 and FoxP4 inhibit HDAC activity. Jurkat T cells transduced with vector or myc-tagged forkhead family members show reduced HDAC activity by fluorescence deacetylation activity. Equal numbers of cells were incubated 1 hour with fluorescent cell permeable deacetylase substrate and assayed for intracellular deacetylase activity. Experiment done in triplicate.
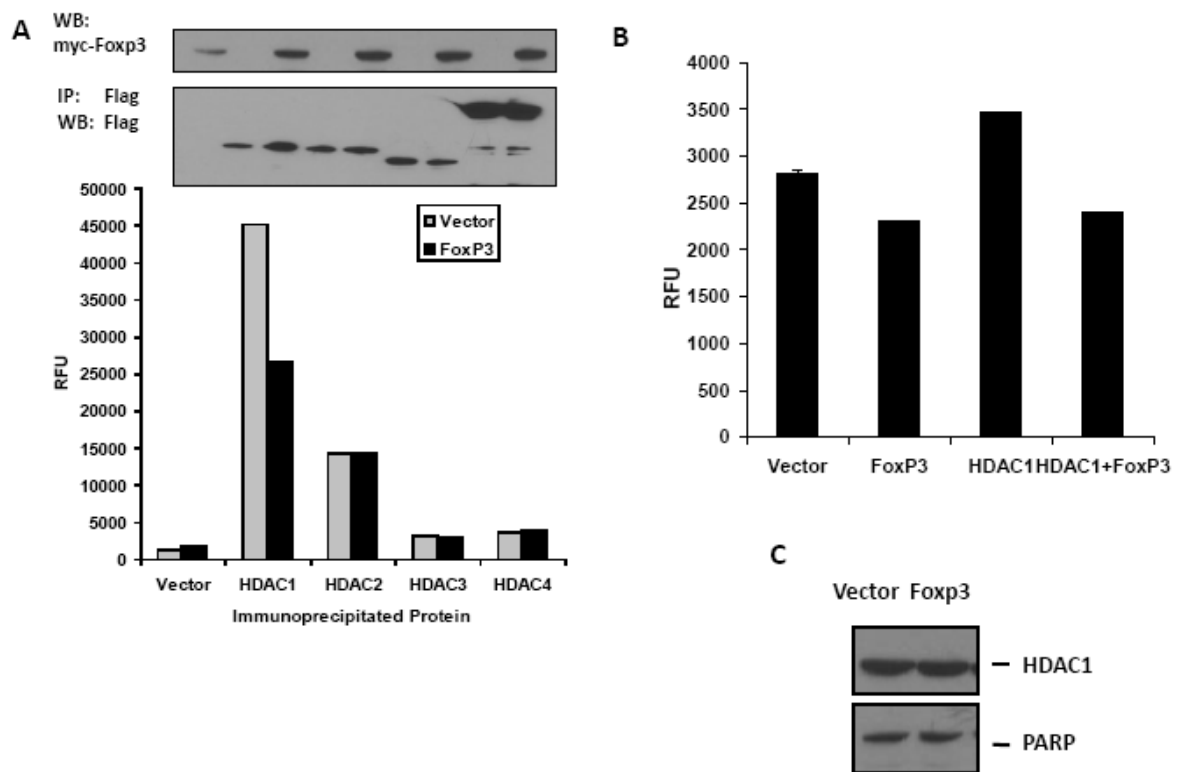
Figure 3.4. N-terminus and Forkhead domains of FoxP3 are required to inhibit HDAC1 deacetylase activity.

(A) FoxP3 inhibits HDAC1 activity. Flag tagged HDAC1-4 are cotransfected into 293T cells with vector or FoxP3. HDAC1 activity is specifically inhibited by Foxp3 coexpression. **(B)** FoxP3 inhibits HDAC 1 *in vivo.* Jurkat cells transduced with vector or Foxp3 in the presence or absence of HDAC1 overexpression were analyzed for HDAC activity. **(C)** HDAC1 levels are unchanged by FoxP3. Nuclear lysates of Jurkats transduced with vector or Foxp3 are assayed for HDAC1 protein levels by western blot. 10ug nuclear lysates are subject to western blot, and probed for HDAC1 and PARP. **(D)** N-terminus and Forkhead domain of FoxP3 are required for HDAC1 inhibition. Foxp3 deletion mutants and point mutants that are unable to inhibit IL-2 gene expression are cotransfected with Flag tagged HDAC1, and HDAC1 deacetylase activity is measured by fluorescence. Data representative of multiple Immunoprecipitations. **(E)**   FoxP3 mutant lacking repressive ability are unable to inhibit HDAC activity. Foxp3 and WWRR transduced Jurkat T cells are incubated with cell permeable HDAC substrate and assayed for HDAC activity.
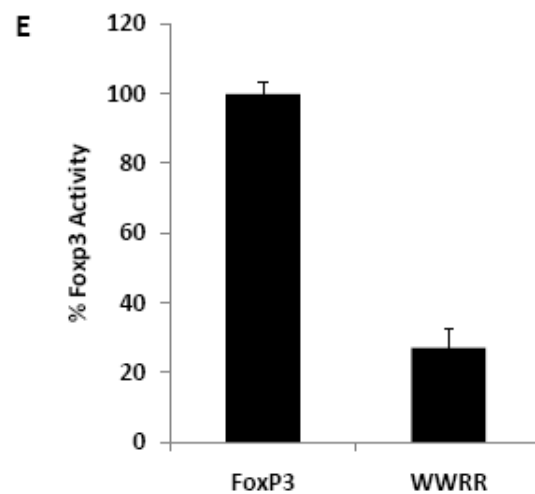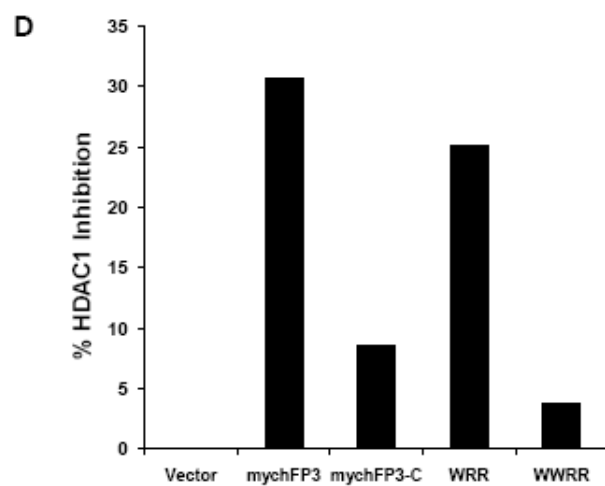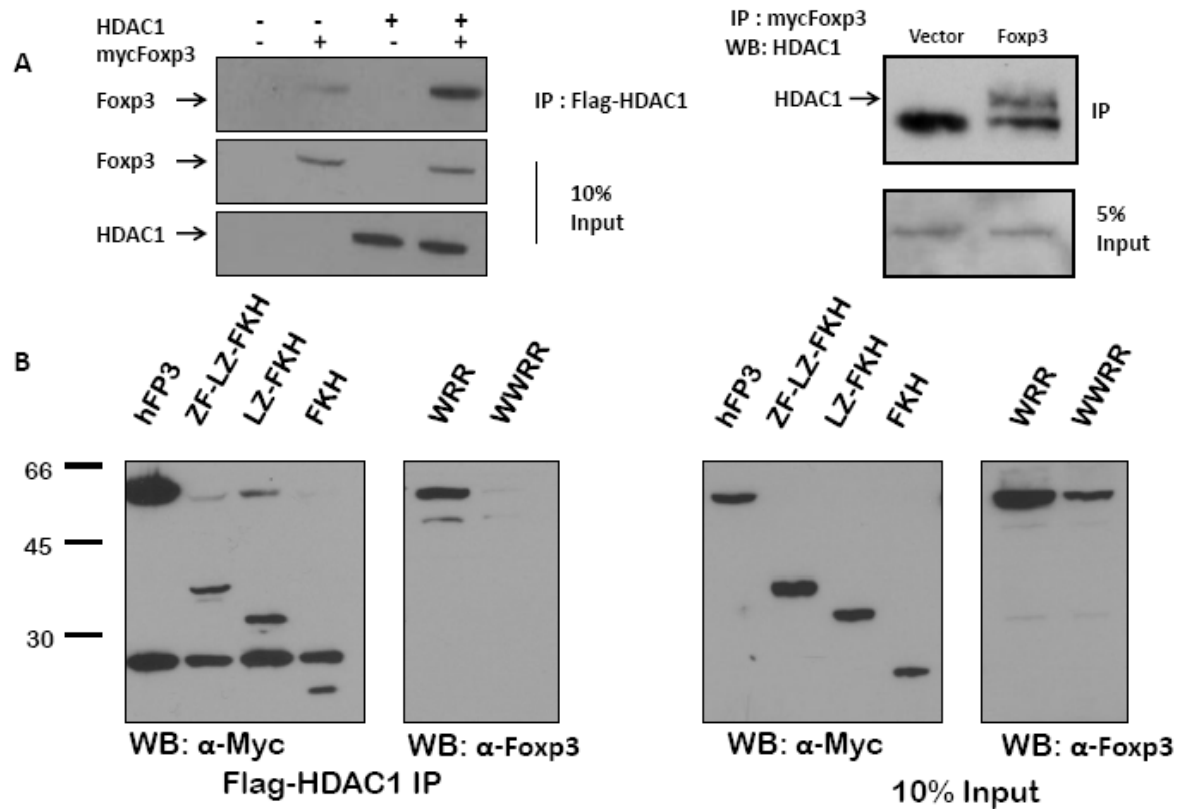
Figure 3.5. FoxP3 interacts with HDAC1.

(A) 293T cells cotransfected with flag-HDAC1 and myc-tagged FoxP3 were Immunoprecipitated with anti-Flag antibody and washed under low stringent conditions (*Left*) while Jurkat cells transduced with FoxP3 were Immunoprecipitated with myc9E10 antibody and probed for endogenous HDAC1 (*Right*). **(B)** Forkhead mutation ablates Foxp3:HDAC1 interaction. 293T cells were transfected with myc-FoxP3 deletion mutants and Flag-HDAC1. HDAC1 was immunoprecipitated with anti-Flag M2 antibody and visualized by western blot.

Figure 3.6. HDAC1shRNA reduces Foxp3 activity at the *IL-2* and HIV-1 LTR promoters.

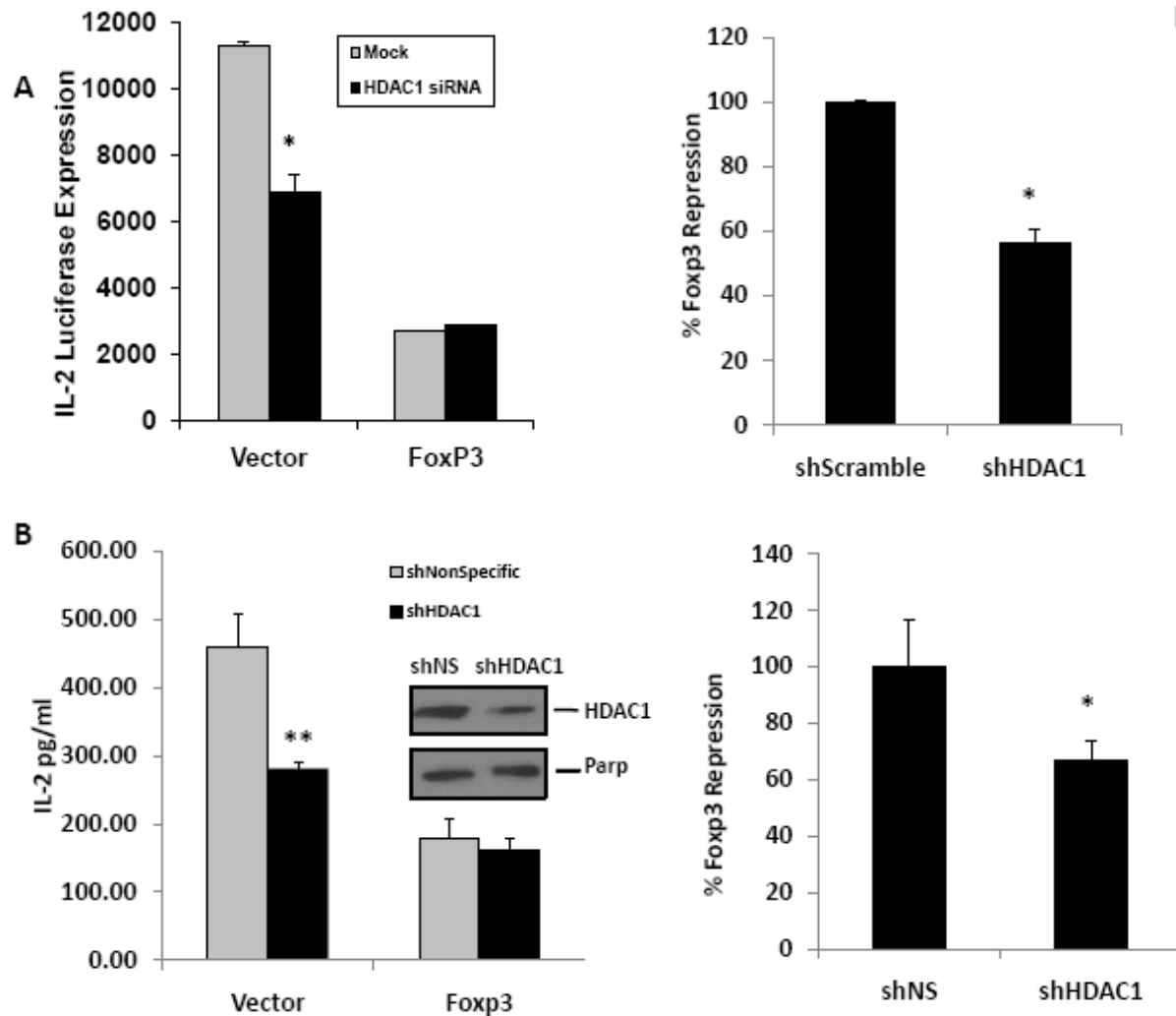**(A)** Jurkat T cells were transfected with siRNA targeted to HDAC1 or a scrambled siRNA as a control, and cotransfected with IL-2-luciferase plasmid. 24 hours post transfection, T cells were activated with PMA/Ionomycin. 48 hours post transfection cells were lysed and luciferase activity was measured. Activity is normalized to GFP expressing cells. **(B)** Endogenous *IL-2* expression was measured in Jurkat T cells stably expressing shRNA specific for HDAC1 or a non-specific shRNA (NS) transduced with vector or Foxp3 expressing retrovirus. IL-2 secreted into the media was detected by ELISA. (Inset) Western blot showing knockdown of endogenous HDAC1 in stably transduced Jurkat T cells. **(C)** Similar Jurkat T cells transduced with shHDAC1 or shNS lentivirus in the presence or absence of FoxP3 were infected with NL-4 luciferase virus and gene expression was measured my luciferase expression. Results shown are representative experiments done in triplicate. * p<.05, ** p<.005 using student *t*-test.

Jurkat Transduced Extracts
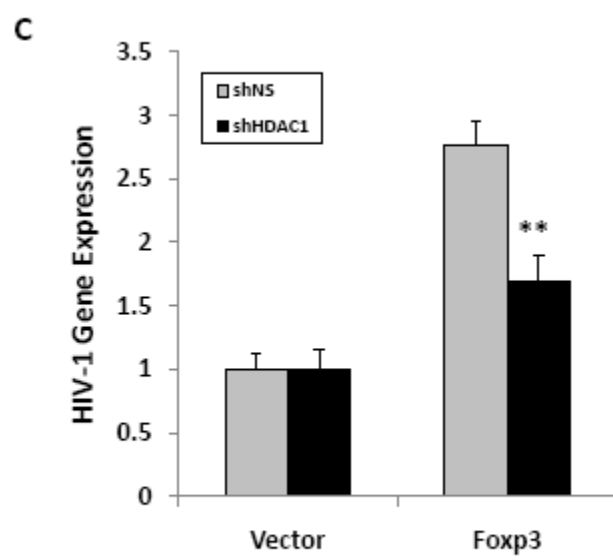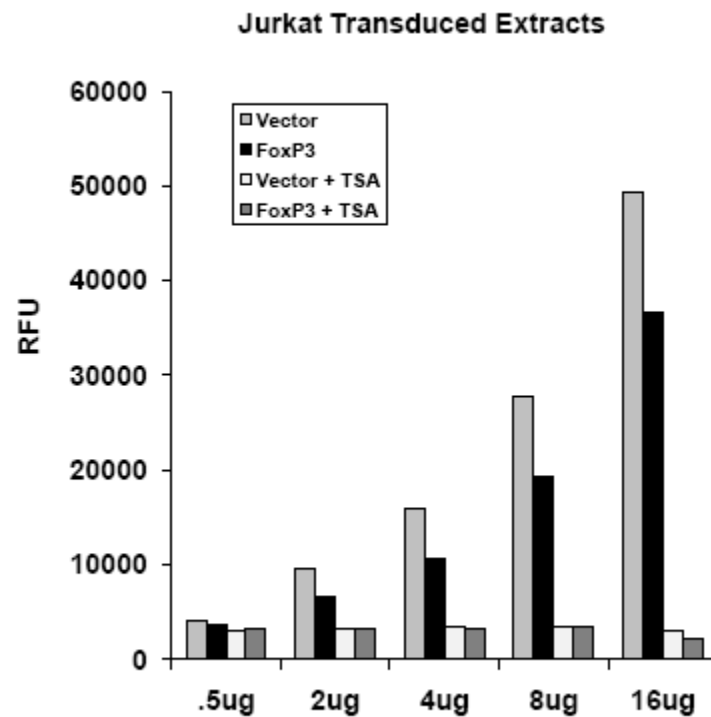
Figure S3.1. HDAC Deacetylase activity in nuclear lysates of Jurkat T cells.

Jurkat T cells transduced with empty vector or FoxP3 expressing retrovirus are separated into nuclear and cytoplasmic fractions. Equal volume of protein was resuspended in HDAC assay buffer and HDAC activity was measured using a fluorometer.

293T cell Size Fractionation
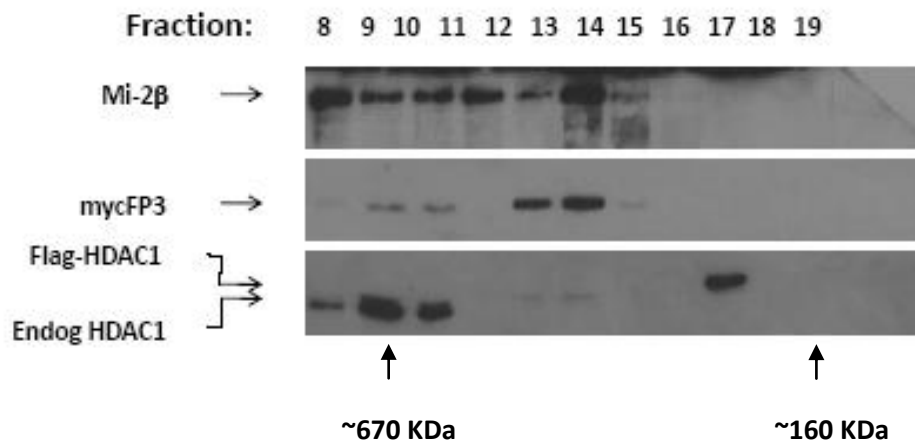
Figure S3.2. FoxP3 and HDAC1 co-fractionate in a high molecular weight complex.

293T cells were cotransfected with FoxP3 and HDAC1. Nuclear lysates were separated and fractionated by size-exclusion gel filtration chromatography column and fractions were resolved on a SDS gel. Antibodies for myc (FoxP3), HDAC1 and Mi-2β were used for analysis by immunoblotting.

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Chapter IV

FoxP3 Regulates IL-2 Gene Expression by Inhibiting Mi-2β Recruitment to the Proximal Promoter in Human T cells.

Derek Holmes and Lishan Su

Abstract

IL-2 plays a critical role in T cell proliferation and survival and is rapidly induced following T cell receptor (TCR) engagement. The current model of transcription initiation at the *IL-2* promoter includes a rapid loss of histones at the proximal promoter within 2 hours of stimulation, followed by recruitment of transcription factors required for optimal gene expression. Little is known about the mechanism of nucleosome remodeling at the *IL-2* promoter, but there is a clear requirement for chromatin remodeling factors in this process. Here, we demonstrate that FoxP3, a member of the forkhead winged-helix family, co-fractionates with a high molecular weight complex that includes the ATP-dependent nucleosome remodeler Mi-2β. FoxP3 is interacts with Mi-2β in T cells, and this interaction requires the zinc finger domain of Foxp3. Using chromatin immunoprecipitation, we demonstrate that Mi-2β is bound to the *IL-2* proximal promoter in unstimulated cells, and remains associated with the promoter following T cell activation. Interestingly, FoxP3 expression is associated with decreased Mi-2β binding to the *IL-2* promoter. Knockdown of Mi-2β by shRNA inhibits *IL-2* transcriptional activity from a proximal promoter, suggesting a role for Mi-2β as a factor required for *IL-*2 activation. Thus, we are the first to report a role for an ATP-dependent nucleosome remodeling factor in the activation, and FoxP3-mediated repression, of the *IL-2* promoter.

Introduction

In the immune system, the role of CD4+CD25+ regulatory T cells (Treg) is central to maintaining self tolerance and immune balance. The hallmark of Treg cells is a lack of *IL-2* gene expression and hypo-proliferation *in vitro* (1, 2), the former a cytokine critical for T cell activation and proliferation. T cell activation through TCR engagement results in a signal transduction cascade resulting in activation of a broad range of transcription factors required for robust proliferation. Initially, an ~300bp region upstream of the transcriptional start site was deemed sufficient for IL-2 activation in the cloned human T cell line Jurkat (3, 4). This promoter/enhancer region contains binding sites for key transcription factors required for optimal *IL-2* expression, namely AP-1, NFAT and NF-κB among others (5).

Rapid induction of *IL-2* upon antigen stimulation requires a coordinated effort of transcription factor binding in a large enhanceosome-like complex (6, 7). Preceded by this is a requirement for chromatin modification resulting in changes in chromatin structure across the *IL-2* promoter. Several studies have shown that in both mice and human naïve T cells, the proximal promoter is inaccessible to digestion by DNase 1 or restriction enzymes. Immediately following stimulation, chromatin accessibility to DNase appears, suggesting a role of chromatin remodeling (5, 8-10), and later studies described a positioned nucleosome in the promoter/enhancer that disassembles following T cell activation (11, 12). Interestingly, Su *et al.* demonstrated that murine Treg cells maintained a relative inaccessibility to MNase digestion at the *IL-2* promoter following activation, and therefore

failed to undergo chromatin remodeling (13). Thus, factors permitting chromatin changes

are important for complete *IL-2* gene expression in T cells and Treg cells.

In eukaryotic cells, mechanisms are in place to repress transcription of genes until an

external or internal response causes gene activation. Histone modifying enzymes and also

ATP-dependent chromatin modifying complexes play a crucial role in unraveling or relaxing

tightly bound chromatin to allow access to transcription factors (14-17). Covalent

modifications of core histone tails include site-specific acetylation, phosphorylation,

ubiquitination and methylation (15, 18, 19). While covalent modifications can change the

chromatin structure at promoters, they are not sufficient to promote histone displacement

and therefore require ATP-dependent chromatin remodeling enzymes.

Two of the most widely studied chromatin remodeling complexes in eukaryotic cells

are the SWI/SNF and Mi-2/NuRD complexes. The NuRD complex was the first complex to

contain both nucleosome remodeling and histone deacetylase functions, linking these two

important chromatin remodeling activities. The multisubunit complex contains a

chromatin remodeling ATPase, Mi-2β, along with HDAC1 and HDAC2 (20-23). Since this

large molecular weight complex contains deacetylase activity, the Mi-2/NuRD complex was

implicated in gene repression. Several studies described transcription factors that associate

with the Mi-2/NuRD complex and target this complex to specific promoters to repress

transcription, such as ikaros and Bcl-6 (24, 25). Despite strong data supporting the role of

Mi-2β in repression, Williams *et al*. described a function for Mi-2β as a positive regulator of

CD4 gene expression in T cell development. In this study, Mi-2β regulated *CD4* promoter by

recruiting the histone acetyltransferase (HAT) p300 to enhance transcription. Still, others

have shown an association of Mi-2β with RET Finger Protein (RFP) and Brg1 biochemically,

resulting in either repression or activation of a targeted promoter, respectively (26). Thus,

ATP-dependent chromatin remodeling factors are required for differential regulation of

gene transcription by multiple mechanisms.

The mechanism of gene regulation by FoxP3 in Treg cells has been of great interest

in the past decade. FoxP3 expression in T cells results in both upregulation and repression

of several genes in Treg cells and is thought to be a lineage specific factor (27-30).

Mechanistically, FoxP3 has been shown to repress or activate genes through regulation of

transcription factors. NFAT and NF-κB transactivation function is regulated by FoxP3 (31-

34), and Wu *et al.* have shown that FoxP3 inhibits the binding of NFAT/AP-1 at the promoter

enhancer of *IL-2* (32). Still others have shown an association of FoxP3 targeted promoters

with either an increase or decrease in histone acetylation on activated or repressed genes,

respectively (35). Interestingly, FoxP3 has been shown to associate with a large molecular

complex in T cells that includes chromatin modifying factors (36), and Li *et al*. described a

HAT/HDAC complex required for FoxP3-mediated *IL-2* repression (37). Therefore, evidence

for a requirement of chromatin remodeling factors for FoxP3-mediated repression of *IL-2* is

abundant.

In this report, we define a role for FoxP3 and Mi-2β in *IL-2* gene regulation. We

demonstrate that Foxp3 and Mi-2β co-fractionate to a high molecular weight complex, and

that FoxP3 associates with Mi-2β Jurkat T cells and 293 cells, with requires the zinc finger

domain of FoxP3 for interaction. We also demonstrate that FoxP3 protein expression is

associated with a decrease in Mi-2β binding at the *IL-2* promoter by chromatin

immunoprecipitation. Conversely, a promoter activated by FoxP3, *CD25,* has no change or a

slight increase in Mi-2β binding. We also define a role for Mi-2β as a factor required for

optimal activation of IL-2 promoter by shRNA knockdown of Mi-2β. Thus, we report a

function for FoxP3 in the regulation of *IL-2* through inhibition of chromatin remodeling

factors required for promoter activation.

Results

FoxP3 co-fractionates with Mi-2β in a high molecular weight complex. Previous

studies have implicated FoxP3 as a regulator of transcriptional activity by association with

high molecular weight complexes. Namely, Li *et al.* determined that FoxP3 complexes with

Brg-1 and MBD3, major components of the SWI/SNF and Mi-2/NuRD complexes (36).

Previous data from our lab demonstrated FoxP3 associates in human T cells with HDAC1, a

constituent of the Mi-2/NuRD complex. We therefore wanted to determine the role Mi-2β

plays in FoxP3-mediated transcriptional regulation. 293T cells were transfected with myc-

tagged FoxP3 or vector control and nuclear extracts were subjected to size-exclusion

chromatography (See materials and methods). FoxP3 eluted in multiple high molecular

weight fractions, ranging in size from >670 KDa to 400 KDa, and co-fractionated with Mi-2β

(Fig. 4.1). Similarly, other NuRD components including MBD2 and HDAC1 were also

localized in these fractions (Data not shown), pointing to a multi-subunit complex that

includes FoxP3 and NuRD proteins.

**FoxP3 and Mi-2β interact in T cells and 293 cells, and the Zinc-Finger domain of

FoxP3 is required for binding.** We next wanted to determine if Foxp3 is able to associate

with endogenous Mi-2β. 293T cells transfected with myc-FoxP3 or vector control plasmid

were immunoprecipitated with myc antibody 9E10 and probed for the association of

endogenous Mi-2β. Similarly, human Jurkat T cells transduced with retrovirus expressing

FoxP3 or empty control retrovirus were fractionated and lysed, followed by immunoprecipitation with anti-myc antibody. FoxP3 was able to efficiently bind endogenous Mi-2β in 293T cells and Jurkat T cells (Fig. 4.2A). To determine the domain of FoxP3 required to interact with Mi-2β, 293T cells were cotransfected with Flag-Mi-2β and myc-tagged N-terminal deletion mutants lacking the N-terminal proline-rich domain (FoxP3-C) only, lacking the N-terminus and zinc finger domains (FoxP3-LZ-FKH), or FoxP3 containing just the forkhead domain (FoxP3-FKH). Immunoprecipitation of Mi-2β with anti-Flag M2 antibody was able to co-precipitate full length FoxP3 and the mutant lacking the proline-rich region (FoxP3-C), but was unable to interact with FoxP3 lacking the zinc finger domain (Fig. 4.2B). Therefore, FoxP3 binds Mi-2β in 293T cells and Jurkat T cells, and the interaction requires the zinc finger domain.

Mi-2β occupies the promoter/enhancer region of *IL-2,* and FoxP3 expression is associated with a decrease in Mi-2β binding. Mi-2β is an ATP-dependent nucleosome remodeling factor and therefore functions to modify the chromatin structure at promoters. More importantly, Mi-2β is a member of the multi-subunit complex Mi-2/NuRD, which has been previously shown to associate with HDACs to repress promoter transcription. Previous studies have demonstrated the requirements of ATPase-containing chromatin remodeling complexes in the activation and inhibition of gene expression (38). Therefore we wanted to determine the function of Mi-2β at the *IL-2* promoter. We chose to analyze the ability of Mi-2β to occupy the enhancer region of the *IL-2* promoter by chromatin

immunoprecipitation (ChIP) assay. This region contains the FoxP3/NFAT binding site required for *IL-2* repression by FoxP3, and is located near a region that undergoes nucleosome remodeling following T cell activation (11). Jurkat cells transduced with retrovirus expressing FoxP3 or vector control were lysed and subjected to ChIP analysis following various times of stimulation. Mi-2β was present at the *IL-2* proximal promoter in unstimulated vector transduced Jurkat cells and maintained association or was slightly increased after 6 hours of activation with PMA and Ionomycin. In FoxP3 expressing cells, Mi-2β was significantly reduced at the *IL-2* promoter in unstimulated cells and following stimulation (Fig. 4.3A). At a later timepoint, 12 hours post activation, Mi-2β was reduced 2 fold at the promoter, indicating a requirement for continual Mi-2β binding during stimulation (Fig. 4.3B). Interestingly, the association of another NuRD component, HDAC1, was unchanged in FoxP3 expressing cells (Fig. 4.3B), potentially excluding a function for the NuRD complex in *IL-2* regulation. Thus, Mi-2β could be functioning in a capacity independent of both the NuRD complex and HDAC1, and rather as an activating factor at the *IL-2* promoter.

We also wanted to determine the ability of Mi-2β to associate with another promoter known to bind FoxP3. *CD25*, a promoter that is differentially regulated by Foxp3, is enhanced by the expression of FoxP3 in T cells. Therefore we wanted to determine the binding of Mi-2β at a promoter upregulated by FoxP3 in Treg cells. Under activation conditions in which *IL-2* is repressed and Mi-2β binding to the promoter is decrease in FoxP3 expressing Jurkat T cells, Mi-2β was bound to the CD25 promoter and FoxP3 expression was associated with no change or a slight enhancement in Mi-2β binding (Fig.

4.4). Thus, Mi-2β association with promoters is differentially regulated in Foxp3 expressing T cells.

Knockdown of Mi-2β in human T cells inhibits *IL-2* gene expression. Previous studies have alluded to a function of chromatin remodeling factors in the inhibition of *IL-2* gene expression in Treg cells. Upon CD3 and CD28 stimulation, the *IL-2* promoter shifts from a closed to an open chromatin conformation as demonstrated by an increase in chromatin accessibility micrococcal nuclease accessibility assays, and the accessibility of the *IL-2* promoter is reduced in Tregs (13). Several ATP-dependent chromatin remodeling factors are located at promoter regulatory regions and are required for relaxing the chromatin to allow for transcription factor binding and promoter activation. The above ChIP data points to a possible requirement for Mi-2β in *IL-2* gene expression. Therefore, we wanted to determine if Mi-2β is capable of modulating *IL-2* activation. Jurkat T cells were transfected with an IL-2-luciferase expression plasmid in the presence of a non-specific shRNA (NS) or a Mi-2β-specific shRNA expression plasmid. Knockdown of Mi-2β inhibited the activation of IL-2 promoter expression by 2 fold compared to NS control (Fig. 4.5). Therefore, IL-2 requires Mi-2β function for optimal gene expression in T cells.

Discussion

In this report we study the interaction of FoxP3 and the chromatin remodeling factor Mi-2β in human T cells. Reported here and previously from the Mark Greene's group (36) it has been shown that FoxP3 protein is found in a low (not described here) and high molecular weight complex by fractionation based on size exclusion gel filtration of nuclear extracts. In molecular weight fractions ranging from 420 to >670, FoxP3 co-fractionates with the ATP-dependent chromatin remodeling factor Mi-2β. Importantly, we demonstrate a strong interaction between FoxP3 and Mi-2β in both nuclear lysates of transient transfected 293T cells and Jurkat T cells expressing FoxP3 by retroviral transduction. We show that this interaction requires the zinc finger domain of FoxP3. To define the function of Mi-2β in T cell activation, we demonstrate that in the presence of FoxP3 protein, Mi-2β association with the *IL-2* proximal promoter is reduced 2 fold at multiple timepoints following strong stimulation with PMA and Ionomycin. Interestingly, at a promoter that is enhanced by FoxP3 expression, *CD25*, there is a slight increase in Mi-2β binding when FoxP3 is present. Finally, knockdown of Mi-2β in Jurkat T cells by transfection resulted in a decrease in IL-2 expression by an IL-2 proximal promoter driving luciferase reporter, pointing towards a function for Mi-2β in activation of IL-2 transcription.

As a model for T cell activation, the *IL-2* promoter has been studied extensively. In naïve T cells, the *IL-2* promoter is maintained in a compacted, closed chromatin conformation. Following TCR ligation or phorbol stimulation (PMA), the nucleosome

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spanning the core enhancer on the *IL-2* promoter disassociates to allow transcription factor binding. It has been proposed at the IL-2 promoter that nucleosome disassembly following activation is maintained for the duration of the external activation signal, and can be reversed following the removal of stimuli (11). A study from the Smale group addressed the mechanism of activation of inflammatory response genes in macrophages following LPS stimulation. Two chromatin remodeling complexes, SWI/SNF and Mi-2/NuRD, associated with multiple promoters and had antagonistic function. The SWI/SNF complex was found to be constitutively present at the primary response promoters such as *Ccl5* and *ifnb1*(38), and studies with IFN-α inducible promoters report a model wherein rapid transcription induction is required by the association of bound ATP-dependent chromatin remodeling factors (39). A similar model of promoter occupancy by Mi-2β could occur at the *IL-2* promoter, where the association of Mi-2β ATPase is required for nucleosome disassociation and transcription factor binding. SWI/SNF is bound to primary response promoters in macrophage cells prior to activation, although loss of BRG1/BRM by retroviral knockdown had no effect on the activation of early primary response genes. Based on our Mi-2β knockdown experiments, it appears that Mi-2β is required for normal activation of *IL-2*, although the exact mechanism will have to be determined.

At the present time, it is unclear if the reduction of Mi-2β at the *IL-2* promoter requires the interaction between FoxP3 and Mi-2β. One could envision a model where FoxP3 protein expression results in the binding of Mi-2β and subsequent removal from the *IL*-2 promoter, thus functioning to actively remove factors from a promoter. A more reasonable model might include a more indirect function of FoxP3. Promoters that FoxP3

binds to and activates, such as CD25, showed a slight increase in Mi-2β association. FoxP3 could actively recruit Mi-2β to enhance transcription, redistributing a limited pool of chromatin remodeling factors other promoters. Proper activation of the *CD4* gene requires a Mi-2β/p300 HAT complex to activate transcription, therefore FoxP3 might recruit an activating Mi-2β/HAT complex to promoters that are directly enhanced by FoxP3 association.   Conversely, FoxP3 might recruit other chromatin modifying factors to the *IL-2* promoter, resulting in displacement of Mi-2β. This latter model would make the protein-protein interaction inconsequential with respect to the *IL-2* promoter. Future studies will be needed to determine what chromatin modifying complexes are present at the *IL-2* promoter, and if FoxP3 actively recruits a repressive complex. We have yet to determine the effect of Mi-2β on FoxP3 regulation of targeted promoters. Mi-2β stable knockdown in FoxP3 expressing cells will allow us to determine to function of Mi-2β in FoxP3-mediated promoter regulation.

The functional consequence of the FoxP3/ Mi-2β interaction with respect to Treg function has yet to be determined. The question remains if the suppressive function or *in vitro* proliferation defect relies on the interaction of FoxP3. Previous reports using a conditional Mi-2β knockout in T cells showed a requirement for Mi-2β in *CD4* promoter regulation. Interestingly, it was noted that peripheral T cells lacking Mi-2β showed a defect in *in vitro* proliferation, a function that was independent of TCR engagement since PMA and ionomycin activation showed similar results as CD3/CD28 engagement. Thus, the function of Mi-2β, and more importantly the FoxP3/ Mi-2β interaction, in Treg hypoproliferation should be determined.

It is unclear if FoxP3 expression in Jurkat cells will result in the decreased chromatin accessibility seen in primary Tregs activated *in vitro*. It will be crucial to demonstrate that FoxP3 reduces MNase digestion in Jurkat cells, and similarly, if Mi-2β is required for chromatin remodeling at the *IL-2* promoter. Whether Mi-2β shRNA-mediated knockdown will result in impaired nucleosome remodeling will have to be determined, either by MNase digestion activity or assessment of histone acetylation dynamics at the proximal *IL-2* promoter.

To date, FoxP3 has been shown to interact with proteins ranging from transcription factors such as NFAT, NF-κB and Runx1, to chromatin remodeling factors such as the HAT Tip60, HDAC1, 7 and HDAC9. FoxP3 is organized into four structural domains, which includes the most N-terminal proline-rich region, zinc finger domain, leucine zipper, and C-terminal forkhead domain. The forkhead domain has a clear function in both DNA binding and transcription factor interaction, and the N-terminus is required for transcriptional regulation. So far, natural mutations of FoxP3 in IPEX individuals have been found in every region but the zinc finger domain. As a functional domain, the zinc finger and the leucine zipper are the minimal requirements for FoxP3 homo- and hetero-multimerization, although point mutations that disrupts the zinc finger structure are unable to inhibit multimerization (36). This data along with the lack of natural mutations in this region found in IPEX patients makes the role of the zinc finger in FoxP3 function unclear at this time. Therefore, this has been the first description of a FoxP3-protein interaction requiring the zinc finger. Future experiments utilizing zinc finger mutations that inhibit the interaction

between FoxP3 and Mi-2β will provide insight into the functional importance of this specific

interaction.

Materials and Methods

Plasmids, cell lines and shRNA. Jurkat cells were maintained in RPMI 1640 (Gibco BRL), 293T cells were maintained in DMEM and primary human T cells were maintained in Iscoves MEM, supplemented with 10% FBS (Sigma), 2mM L-glutamine, $100\,\text{U/ml}$ penicillin, and $100\,\text{mg/ml}$ Streptomycin. Mi-2β shRNA expressed from a lentivirus vector pLKO was obtained from Open Biosystems (Open Biosystems). Foxp3 expressed in HSPG retroviral vector was previously described (40).

Gel Filtration Chromatography. Nuclear lysates isolated from 293T cells were filtered through Ultrafree-MC 0.22μm columns prior to chromatography. 0.5 mg of total protein was run on a Superose 6 10/300GL (Amersham Bioscience) gel filtration column using a Duoflow chromatography system (Bio-Rad) in PBS with a flowrate of 0.3ml/min. Fractions were collected in 2ml aliquots and subjected to analysis by western blot.

Transfection of Jurkat T cells and luciferase assay. Jurkat cells were transfected by electroporation. Briefly, 1x10^6 cells were incubated with 15ug empty vector or Foxp3 and 1ug IL-2 luciferase plasmid along with 500ng of a control GFP plasmid. 24 hours post transfection, Jurkat cells were activated with PMA (25ng/ml) and Ionomycin (1um) for 24 hours. Cells were lysed in 1x reporter lysis buffer (promega) and luciferase expression was

determined by Luciferase Assay System (Promega). Luciferase readings were normalized to cell number and % GFP expression for every sample by Guava. Experiments were done in triplicates and repeated at least three times.

293T cell transfection, Immunoprecipitation and Western Blot. 293T cells were transfected using Effectene transfection reagent (Qiagen) per manufacturer recommendations. Cells were fractionated into cytoplasmic and nuclear lysates and quantitated by BCA protein assay kit (Pierce). 500ug nuclear lysates were incubated overnight with 2ug Flag antibody (Sigma) followed by Protein A bead incubation. For HDAC assay, beads containing immunoprecipitated complex were washed 4X with PBS and HDAC activity was analyzed by Flour De Lys HDAC assay as described below. For IP-western, beads were washed in modified RIPA buffer and subject to SDS-page followed by transfer to PVDF membrane and visualization by Western Blot.   FoxP3 was probed with an anti-FoxP3 antiserum (41) followed by secondary α-rabbit‑IgG‑HRP (1:10,000) (Amersham Biosciences) and visualized by ECL (Amersham Biosciences).

ChIP assays. Jurkat E6.1 cells were transduced with control or FoxP3-expressing vectors. Sonicated chromatin from the Jurkat cells was IP'ed with control IgG (IgG), anti-Mi-2β (Santa Cruz) or anti-HDAC1 (Ebioscience) and assayed by chromatin immunoprecipitation assay kit per manufactures instruction (Upstate, Millipore). IP'ed chromatin was purified using a PCR purification kit (Qiagen) and samples were subject to real-time qPCR analysis by

syber green. The relative Mi-2β binding or HDAC1 binding to the IL-2 enhancer promoter is expressed with ratios of ChIP-specific signal divided by signals from control 10% input chromatins. IL-2 promoter was analyzed with the following primers: 5'-cac cta agt gtg tgg gct aat gta ac-3' and 5'-ctg atg act ctt tgg aat ttc ttt aaa cc-3'. This amplicon spans -226 to -133 in the human IL-2 promoter and spans the NFAT and Foxp3 binding sites.

Statistical analysis. For statistical analysis, a two-tailed Student t-test was employed where P<0.05 was considered significant.

Acknowledgements
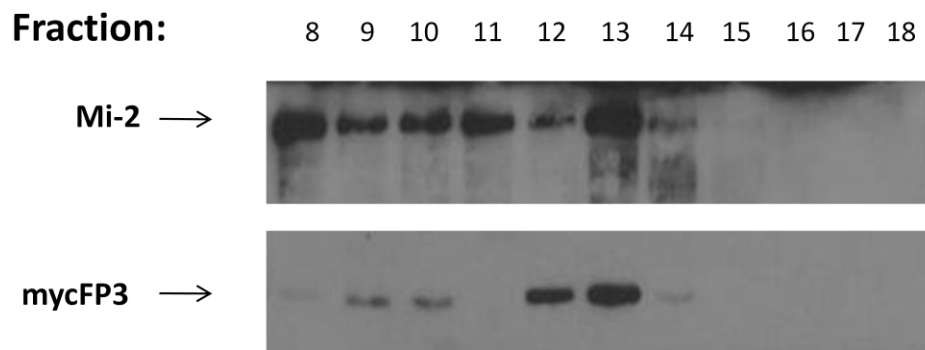
Figure 4.1. FoxP3 localizes with Mi-2β in a high molecular weight complex in 293T cells.

293T cells were transfected with myc-tagged human FoxP3, and 48 hours post transfection nuclear lysates were isolated. FoxP3 complexes were size-fractionated by gel filtration columns followed by western blot analysis with anti-myc antibody and anti-Mi-2β antibody specific for endogenous Mi-2β.
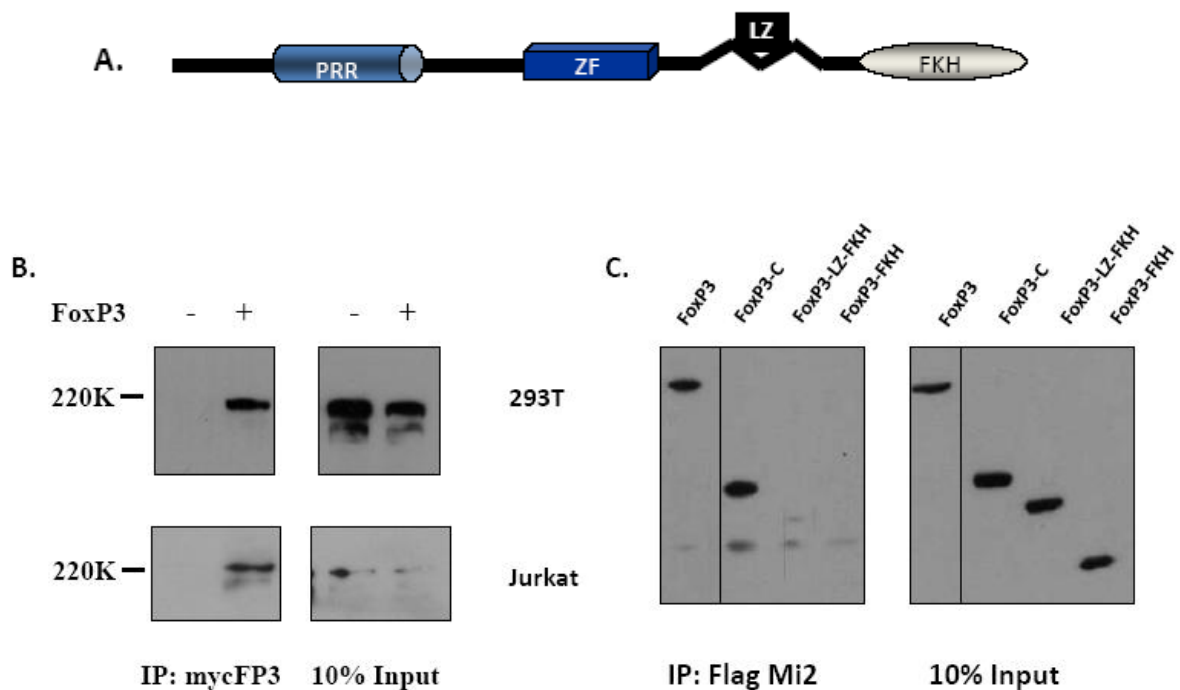
Figure 4.2. FoxP3 interacts with Mi-2β, and requires the zinc finger domain.

(A) Domain organization of Foxp3. Proline-rich region (PRR), zinc-finger domain (ZF), leucine zipper region (LZ) and Forkhead DNA-binding domain (FKH). **(B)** 293T cells were transfected with mycFoxp3 (Top) or Jurkat T cells were transduced with empty vector or Foxp3 expressing retrovirus (Bottom), nuclear lysates were subjected to immunoprecipitation with anti-myc antibody, and visualized by western blot against endogenous Mi-2β. **(C)** 293T cells were cotransfected with myc-tagged Foxp3 or a series of N-terminal truncation mutants deleting the proline-rich region (Foxp3-C), containing the leucine zipper and forkhead only (Foxp3-LZ-FKH), or containing the forkhead domain (Foxp3-FKH) along with flag-tagged Mi-2β.
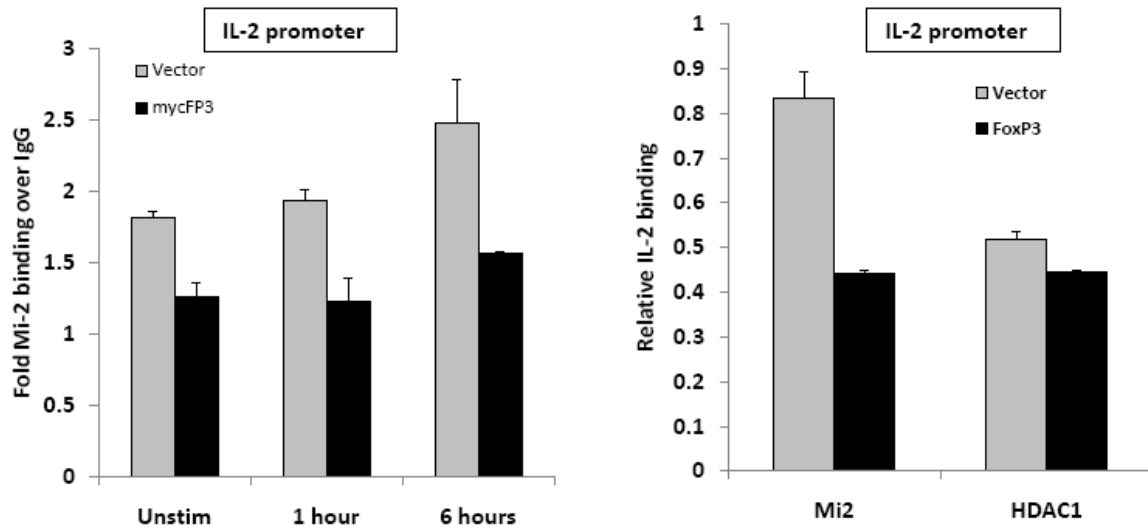
Figure 4.3. Foxp3 regulates Mi-2β association at the *IL-2* promoter in Jurkat T cells.

Jurkat T cells are transduced with vector or Foxp3 and mock stimulated or stimulated for 1 or 6 hours (Left graph) or 12 hours (right graph) with PMA and Ionomycin. Cells are chemically crosslinked, and chromatin is immunoprecipitated with anti-Mi-2 or HDAC1 antibodies. Enrichment of IL-2 proximal promoter is determined by Syber Green quantitative Real-time PCR. Fold enrichment represents signal over IgG. Samples are measured in triplicates for each experiment.
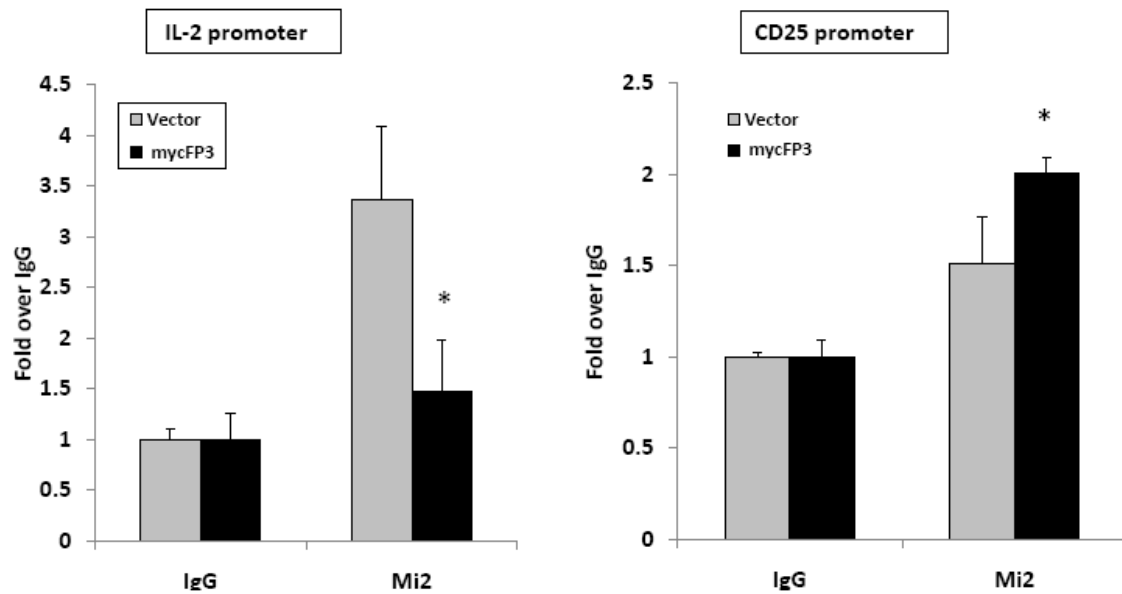
Figure 4.4. Promoters differentially regulated by Foxp3 are associated with differential binding of Mi-2β.

Jurkat T cells transduced with empty vector or Foxp3 are stimulated with PMA and Ionomycin for 12 hours and relative Mi-2β binding at the *IL-2* and *CD25* promoters is determined by ChIP assay. Samples are normalized to 10% input and fold enrichment over IgG is determined.
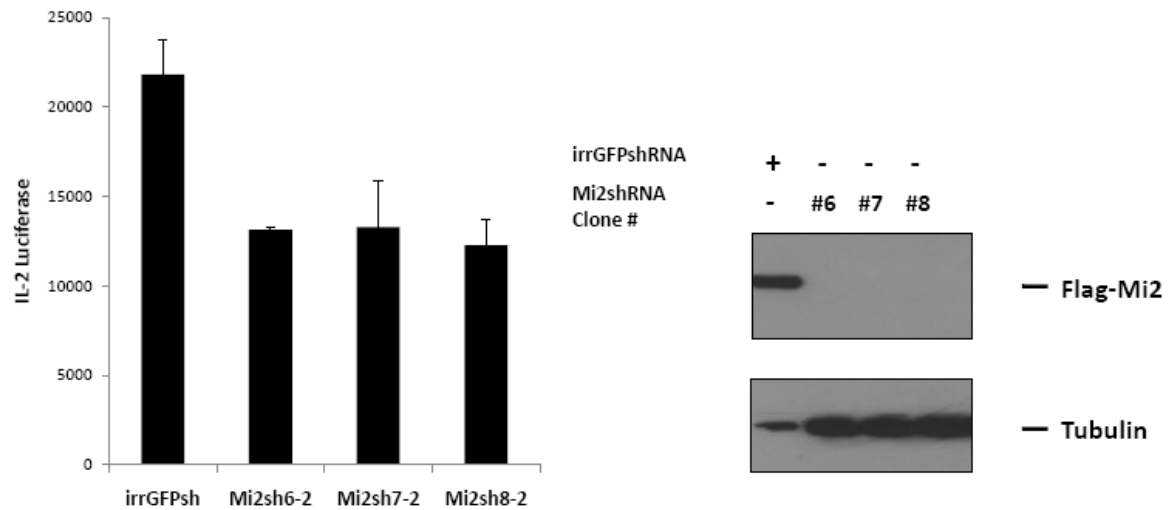
Figure 4.5. Mi-2β is required for optimal IL-2 expression in Jurkat T cells.

Jurkat T cells are transfected with shRNA lentivirus plasmid containing a control irrelevant GFP shRNA or three shRNA plasmids designed against Mi-2β. Knockdown of Mi-2β in 293T cells is demonstrated by western blot against a flag-Mi-2 overexpressed in 293T cells (Left). IL-2 luciferase plasmid is cotransfected with shRNA plasmids in Jurkat T cells and luciferase is determined by luciferase assay. Results are normalized to a GFP reporter for all transfected samples.

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Chapter V

Conclusions and Future Directions

Perspective and Scope

While the role of FoxP3 in the biology of Treg cells is currently being scrutinized, it is clear that an elaborate and poorly understood landscape of gene regulation and cell signaling is required to make a Treg a 'regulatory cell'. The fact that T cells can be guided into a regulatory phenotype at multiple stages of the T cell life cycle, from development of natural Tregs in the thymus to the conversion to adaptive Treg cells in the periphery, highlights the importance of this subset of T cells in the grand view of immune regulation. Through Microarray technology and ChIP-Chip assays, the profile of gene regulation and targets of FoxP3 transcriptional regulation on a genomic scale has been described. Comparisons of gene regulation between general T cell activation versus natural or induced Tregs have narrowed the scope of gene that are directly modulated upon FoxP3 expression in T cells. Thus, the foundation is in place for determining and characterizing the mechanisms of FoxP3 regulation of gene transcription. The scope of this body of work is to do just that, find novel *in* vivo targets of FoxP3 that are critical for transcriptional regulation. Herein, I will summarize findings of novel mechanisms and targets for FoxP3-mediated regulation, the implications of these findings, and future experiments and directions to further our knowledge of FoxP3 function.

Chapter2: FoxP3 enhances HIV-1 LTR through an NF-κB-dependent mechanism.

Findings and Implications

Chapter two highlights a novel function of FoxP3 in the regulation of gene

expression, namely, the activation of HIV-1 LTR in human T cells. Here we describe

disparate regulation of the HIV-1 LTR compared to the well studied and documented *IL-2*

gene. Previous findings at the *IL-2* promoter have described a model wherein FoxP3

regulates promoter activity through modulation of multiple transcription factors. Initially it

was shown that FoxP3 can form a complex with NFAT, effectively inhibiting the formation of

an NFAT/AP-1 complex (1). More recent data have suggested that FoxP3 is also able to

negatively regulate AP-1 DNA binding activity without altering protein levels, further

supporting a role of inhibition of a NFAT/AP-1 complex (2). FoxP3 is also capable of

combining with other transcription factors normally required to activate *IL-2*, as seen with

the Foxp3/Runx1 complex. Here we demonstrate the requirement for yet another

transcription factor, NF-κB, required for FoxP3-mediated activation of HIV-1 LTR. Knocking

down NF-κB or ablating the NF-κB binding sites in the LTR completely inhibits FoxP3-

mediated activation.   We describe a model where FoxP3 enhances the binding of NF-κB at

the LTR, but not the *IL-2* promoter as one mechanism of regulation. Importantly, we

integrate both modulation of transcription factor binding and histone modifications with

differential regulation of LTR and *IL-2*. We also describe differential regulation of histone

acetylation, where FoxP3 repression of *IL-2* is associated with decreased acetylated histone

3, while the LTR activation is associated with an increased in histone acetylation. Consistent

with this result, other groups have described an association of Foxp3 activation or inhibition

of gene expression with increased or decreased acetylated histones, respectively (3).

To date, Foxp3 has been shown to directly and indirectly regulate genes and act as

both an activator and repressor. Studies using T cell hybridomas lacking or expressing

Foxp3, or natural Treg cells versus Foxp3- T cells, analyzed on a global scale the promoters

that were directly bound by Foxp3 (4, 5). Of note, Foxp3 directly bound only a small

fraction of the promoters of genes that were differentially regulated upon *Foxp3*

expression, revealing both a direct and indirect role of Foxp3 in gene regulation.

Interestingly, most of the promoters that Foxp3 directly occupied were (i) responsive to TCR

stimulation, (ii) regulated only upon activation, and (iii) were associated with a NFAT

binding motif. This correlates with the fact that most of the Foxp3 responsive genes are

NFAT-dependent. Regulation of the HIV-1 LTR by FoxP3 is intriguing for the fact that FoxP3

enhances LTR independent of NFAT activation, under unstimulated conditions or upon

CD3/CD28 stimulation, while strong stimulation such as PMA/Ionomycin negates the FoxP3

effect. Marson *et al*. clustered several genes that were differentially regulated +/- Foxp3

and +/- stimulation, and associated these clusters with Foxp3 promoter occupancy.

Interestingly, the gene cluster that mirrored Foxp3 regulation of LTR, that is to say genes

that were activated with and without stimulation in the presence of Foxp3, were associated

with a lack of Foxp3 promoter binding. Preliminary data of FoxP3 binding to the HIV-1 LTR

in 293T cells upon transient transfection followed by ChIP assay shows that FoxP3 is able to

bind the LTR. Thus, HIV might have adapted the LTR to utilize cellular conditions that are optimal for LTR transcription, inconsistent with the functional role of FoxP3 in the regulation of stimulus-dependent gene transcription.

FoxP3 largely plays a similar role in repression between murine and human Treg cells, with one notable difference in the fact that most human T cells activate FoxP3 upon antigenic stimulation, whereas murine Foxp3 is expressed only in Treg cells. Thus, it is thought FoxP3 might play a more general role in the control of the T cell receptor response. This is supported by the fact that Foxp3 dampens genes that are activated upon TCR stimulation. The ability of HIV-1 LTR to respond to FoxP3 might be a direct adaptation of disease pathogenesis and progression. Currently, the topic of Tregs in HIV disease progression has gained considerable attention for the obvious role that Tregs play in T cell activation and function. It is now becoming evident that Treg cells are a direct target of HIV-1, SIV and FIV infection (6-8). Recent analysis of Tregs in SIV infection over time in rhesus macaque model versus sooty mangabey model has given us insight into the potential role of Tregs in disease progression (9, 10). Differences in disease progression between the 'natural' SIV hosts (sooty mangabey, African green monkey) and 'un-natural' hosts (rhesus macaque) highlights the role of overt immune activation as the best predictor of disease progression. The SIV-infected sooty mangabey lacks overt immune-hyperactivation in the presence of high viral load, while rhesus macaque show a decline in CD4+ T cells, hyper-immuneactivation, and disease progression. In support of the critical role of Treg cells in immune-regulation, the differences in Treg dynamics in SIV infection between these two monkey models is important. These studies demonstrate that Treg cells can be depleted in

both acute and chronic stage of SIV infection, although discrepancies have been seen in the former. This decrease in Tregs is similar to the decrease in total CD4+ T cell in the gut, and is associated with an increase in viral load and immune-activation. While Treg function is altered in SIV infected macaques, Treg numbers and function are unchanged in sooty mangabeys, which do not progress towards an AIDS-like syndrome. The conclusion thus far is that HIV and SIV replication is sensitive to Treg cell numbers and function, and a decrease in Tregs over the disease course contributes to overt immune hyperactivation. Data from our lab shows that HIV infection of humanized mice leads to direct infection and depletion of Treg cells. Our finding that HIV infection of Tregs is enhanced by FoxP3 expression supports a model in which preferential infection of Tregs and enhanced HIV-1 gene expression leads to an increase in Treg destruction, leading to loss of T cell activation control and thus an increase in activated T cell targets, allowing for further HIV replication. The Treg might very well be the key to disease progression, the critical cell that acts as both a target and mediator of future targets, and therefore the implication that FoxP3 enhances HIV gene transcription is a crucial component in HIV-1 disease progression.

Future Direction and Studies

There are several obvious future studies and directions related to the enhancement of HIV replication by FoxP3. At the smallest scale, it is still unknown if FoxP3 can directly bind the HIV-1 LTR. As previously stated, ChIP analysis of myc-tagged FoxP3 on the LTR

demonstrates interaction of FoxP3 with the LTR, although ChIP assays utilizing transcription

factor binding site mutants, or successive 5' deletions of the LTR have been inconclusive

thus far. If FoxP3 directly binds the LTR independent of transcription factor-mediated

recruitment, we would expect to find a putative forkhead binding site in the LTR. Thus far,

we have been unable to find a forkhead binding motif in the U3 region to support FoxP3

binding. Conversely, FoxP3 can be recruited to the LTR by transcription factor association.

This model is supported by the finding that FoxP3 can directly bind NFAT and NF-κB to

modulate gene expression. Future studies utilizing ChIP assays to determine FoxP3 binding

to the LTR in the presence or absence of NF-κB would resolve this question. With respect to

enhancement of NF-κB at the LTR, it would be of worth understanding the mechanism of

enhanced binding to the LTR when FoxP3 is expressed. FoxP3 by itself is unable in alter

nuclear localization or retention of NF-κB, therefore it would be interesting to determine if

FoxP3 is somehow able to modify NF-κB post-translationally. One possible way to

determine this would be to immunoprecipitate NF-κB and analyze specific Foxp3-mediated

changes by mass spectrometry. Alternatively, as discussed in chapter 2, the binding affinity

of the NF-κB sites located at the LTR are greater than that of the NF-κB sites located at the

IL-2 promoter. The fact that FoxP3 regulation of *IL-2* is critically NFAT-dependent, the NF-κB

activity is most likely LTR-specific due to the fact that the LTR is highly NF-κB-dependent.

On a much broader scale, it is currently unknown the effect of HIV infection on Treg

function. In SIV infection *in vivo,* the suppressive function of Treg cells is altered. HIV

infection of multiple cells types has been shown to alter cellular signaling and cytokine

expression, therefore the next logical step would be to determine if infected Tregs are still

functional. Does HIV-1 infection change Treg suppressive activity? Can HIV infection alter

cytokine expression, induce apoptosis, or other cellular activities? If so, what are the viral

gene products necessary for modulation of Treg function?   Answering these questions plus

focusing on the role of Tregs in HIV disease progression will define future therapeutic

targets to enhance retroviral therapy in patients.

Chapter 3: FoxP3 inhibits HDAC1 activity to modulate gene expression

Findings and Implications

The third chapter expands upon the notion of FoxP3 as a regulator or transcription

by describing a novel interaction between FoxP3 and the histone deacetylase HDAC1.

Interest into the role of HDAC in FoxP3 regulation stemmed from the finding that FoxP3

activation of HIV-1 LTR was insensitive to TSA. TSA and other HDAC inhibitors have been

shown to activate HIV-1 LTR a histone deacetylation-dependent mechanism. Thus, FoxP3

and HDACi are working through similar pathways to mediate transcription. Therefore, the

question of does FoxP3 work similar to HDACi was first determined by the overexpression of

FoxP3 in Jurkat T cells and analysis of cellular HDAC activity. We demonstrated that FoxP3

expression was able to inhibit global HDAC activity, both upon overexpression of FoxP3 in

Jurkat T cells and in naturally expressing primary Treg cells. We then demonstrated that

FoxP3 inhibited HDAC1 specifically, that FoxP3 and HDAC1 interacted and co-fractionated in similar high molecular weight complexes by gel chromatography. We also showed that knockdown of HDAC1 by shRNA inhibited both FoxP3-mediated regulation of HIV-1 LTR and *IL-2* gene expression. Finally, we demonstrated that *IL-2* promoter activation in T cells requires HDAC1, and that HDAC1 is the likely target of HDACi-mediated *IL-2* repression demonstrated in previous studies.

Class I and Class II HDACs play a versatile role in the regulation of cellular processes, including cell proliferation and survival (class I) and differentiation and maturation (class II). Thus, the use of inhibitors of HDACs (HDACi) has garnered attention for their potent anticancer activity, and increased levels of class I HDACs have been associated with a myriad of cancers (11). Similarly, HDACi has been used in the treatment of graft rejection. Recently, the combination of HDACi and rapamycin (the latter a cell cycle inhibitor and potent immunosuppressive drug) administration decreased inflammatory bowel disease and induced permanent Treg-dependent islet and cardiac allograft survival and donor-specific allograft tolerance in mice (12). Mechanistically, HDACi was able to enhance *in vivo* Treg numbers and function, and was associated with increased FoxP3 expression and acetylation, along with increased inhibitory molecules GITR, PD-1, CTLA-4 and IL-10 in Tregs. Thus, the importance of inhibition of HDAC activity in the suppressive function of Treg cells is undeniable.

FoxP3 inhibition of HDAC1 has important implications in Treg biology and possibly clinical implications. Until recently, the role of individual HDACs in the control of T cell

function and, more importantly, Treg function, was relatively understudied. Within the last

year, the Greene group demonstrated a role for HDAC7 in conjunction with Tip60 and FoxP3

as a required complex in FoxP3-mediated *IL-2* repression (13). In HDAC9^{-/-} mice, Treg

suppressive function is increased and transduction of HDAC9^{-/-} CD4+25- with Foxp3-

expressing retrovirus increased suppressive function compared to transduced wild type

CD4+25- T cells. Similarly, HDAC2-/- mice exhibited a 2-fold increase in Treg suppressive

function (14). The fact that FoxP3 acts as an HDAC inhibitor in this study gives support to

the enhancement of Treg function by HDACi, and provides evidence for HDAC1 inhibition by

HDACi as a target in *in* vivo enhancement of Treg function. Thus it is clear multiple HDACs

play a role in Treg biology, and until this study the role of HDAC1 in FoxP3-mediated

promoter regulation was unknown.

Future Directions and Studies

The study of FoxP3 as an HDAC1-specific inhibitor is the first observation of this

novel function for the FoxP3 protein. The regulation of HDACs has been well documented,

and a role for multi-subunit complex formation, post-translational modifications, and

transcriptional regulation has been described in the literature. We propose a mechanism

wherein protein-protein interaction, and thus multimerization of FoxP3 and HDAC1, is

required for FoxP3-mediated inhibition of HDAC1. This is supported by the fact that point

mutations inhibiting FoxP3 transcriptional regulatory function in Treg cells also ablates

FoxP3 inhibition of, and interaction with, HDAC1. We cannot rule out other modifications

to HDAC1, and future studies will be required to determine if FoxP3 is able to alter HDAC1

by acetylation, phosphorylation, or other covalent modification. We also did not describe

the domain of HDAC1 necessary for Foxp3 interaction with HDAC1. It will be important to

map this interaction by deletion mutant mapping. Li *et al*. showed that FoxP3 is acetylated

by the HAT Tip60, and that this acetylation is required for FoxP3 function. Does FoxP3

inhibition of HDAC1 lead to an increase in Foxp3 acetylation? This can be assessed by

determining if knockdown of HDAC1 by shRNA is able to alter the acetylation status of

FoxP3. Finally, does HDAC1 knockdown inhibit Treg function? Since HDAC1 knockout mice

are embryonic lethal, conditional HDAC1 knockout mice could be used to assess Treg

function. Alternatively, efficient lentivirus delivery of shRNA specific for HDAC1 into

primary Treg cells followed by *in vitro* Treg suppression assay would determine the role of

HDAC1 in Treg function.

The question still remains as to what is the downstream target of FoxP3-mediated

HDAC1 inhibition. As stated above, FoxP3 inhibition of HDAC1 might lead to an increase in

FoxP3 acetylation. Since inhibition of HDACs is associated with upregulation of targeted

promoters, it is unlikely that histones are a direct target for this function at promoters that

are inhibited by FoxP3, such as the *IL-2* promoter. Rather, HDACs have also been known to

acetylate non-histone targets such as transcription factors. TSA treatment of T cells has

been shown to alter NF-κB function. Therefore it would be of interest to determine if

FoxP3-mediated HDAC1 inhibition results in alteration of NF-κB function. Studies looking at

the transactivation or DNA binding activity of NF-κB following FoxP3 expression and HDAC1

knockdown will be of use.
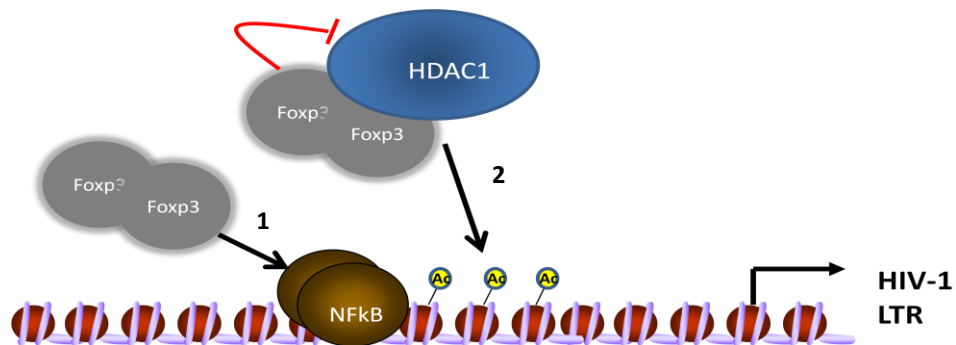
FoxP3 regulation of HIV-1 LTR expression. FoxP3 enhancement of HIV LTR is associated with increased histone acetylation and NF-κB occupancy. (1) FoxP3 enhancement of NF-κB through p65 modification (direct) or inhibition of NFAT1 binding (indirect). (2) FoxP3 inhibition of HDAC1 results in increased acetylation of nuc-1 and enhanced gene expression.
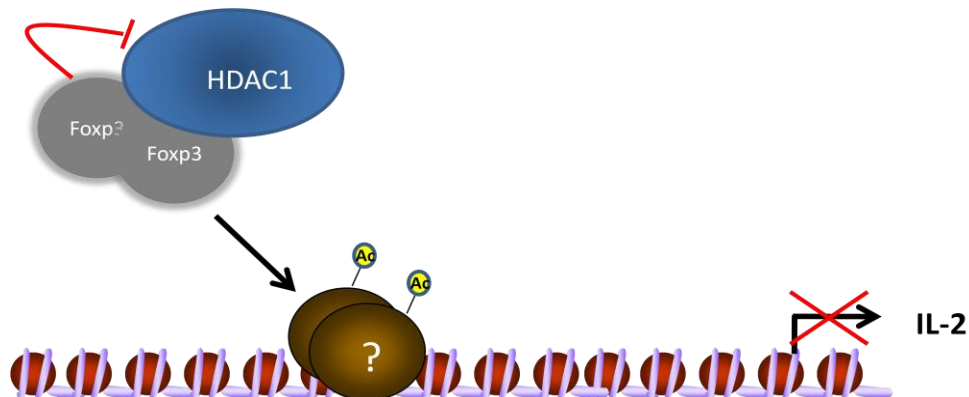
FoxP3 regulation of *IL-2* gene expression. FoxP3 enhancement of *IL*-2 is associated with decreased histone acetylation and no change in NF-κB occupancy. Opposite of HIV-1 LTR, FoxP3 represses *IL*-2 gene expression through inhibition of HDAC1 resulting in hyperacetylation of non-histone factors. Possible targets include NF-κB or NFAT.

Chapter 4: Foxp3 regulates *IL-2* gene expression by inhibiting the ATPase chromatin remodeler Mi-2β.

Finding and Implications

The hypothesis that FoxP3 is capable of regulating promoter activity through multiple mechanisms, from transcription factors to chromatin remodeling dynamics, broadened our scope to include such possibilities. Work from other labs demonstrated that Treg cells have an 'anergic-like' dysfunction *in vitro*, characterized by inhibited *in vitro* cellular proliferation and suppressed cytokine activation. Ultimately, it was demonstrated that the *IL-2* promoter was functionally repressed by inhibition of chromatin accessibility or 'relaxation' of the proximal promoter associated with TCR stimulation (15). This supported the hypothesis of involvement of chromatin remodeling factors in both the normal activation of *IL-2,* but also a Treg-dependent function as well. We initially chose to focus on FoxP3 recruitment of an inhibitory complex, the NuRD complex, as a potential mechanism of FoxP3-mediated repression. In support of this hypothesis, FoxP3 fractionation by gel filtration demonstrated Foxp3 was found in high molecular fractions that included members of chromatin remodeling complexes, namely Brg-1 (SWI/SNF) and MBD3 (NuRD) (16). We similarly showed that FoxP3 overexpressed in 293T cells co-fractionated with both HDAC1

and Mi-2β, thus we set out to describe the interaction of FoxP3 with Mi-2β. We demonstrate that FoxP3 interacts with Mi-2β in Jurkat T cells and 293 cells, and this interaction requires the zinc finger domain of FoxP3. We next determined the role of Mi-2β at the endogenous *IL-2* promoter upon expression of FoxP3 by retroviral transduction. Interestingly, we showed that FoxP3 expression is associated with a loss of Mi-2β near the proximal promoter prior to stimulation and at multiple timepoints thereafter. This did not occurring at a promoter known to be activated by FoxP3, the *CD25* promoter. We hypothesized that Mi-2β might be functioning as a positive regulator of *IL-2* that is required for complete activation. In support of this, we demonstrated knockdown of Mi-2β by shRNA inhibited PMA and Ionomycin activation of *IL-2*-luciferase reporter plasmid. Thus, the conclusion for this study is that FoxP3 alters the binding of a chromatin remodeling factors to FoxP3-regulated promoters. In this case, FoxP3 inhibits Mi-2β required for *IL-2* activation while potentially enhancing the *CD25* promoter.

The NuRD complex is composed of several members that integrate chromatin remodeling with histone deacetylation. Thus, this complex has been described as an inhibitor of promoter activity. Support for the contrary came from a study showing that Mi-2β in T cells is required for *CD4* expression, therefore Mi-2β acts as a positive regulator of *CD4* by recruiting p300 (HAT) to the promoter (17). It is possible that Mi-2β could potentially recruit an activating complex to the *IL-2* promoter as well. The implication of this finding is that (i) there has yet to be described a ATP-dependent nucleosome remodeling factor required for *IL-2* activation, and (ii) FoxP3 inhibition or association with a chromatin remodeling complex with ATP-dependent function has not been previously

described. Importantly, through determination of the molecular interactions between

FoxP3 and other cellular molecules, we are able to gain insight into the fundamental

requirements for *IL-2* activation following T cell stimulation. From this study we can begin

to determine other factors that are perhaps recruited to *IL-2* or other promoters that are

directly regulated by FoxP3.

Future Directions and Studies

Little is known about the role of chromatin remodeling factors that regulate the

nucleosome remodeling or histone acetylation and dynamics at the *IL-2* promoter. The HAT

p300 has been shown to be recruited by factors and is required for proper *IL-2* activation.

Given the enhanced accessibility of the *IL-2* promoter following TCR stimulation, there is a

clear need for determining the ATPase-dependent chromatin remodelers that function at

this and other promoters that respond to TCR signaling. In Treg cells, FoxP3 is responsible

for both the upregulation and downregulation of several genes. The ability of FoxP3 to bind

to and target these promoters suggests, along with data suggesting that FoxP3 is found with

multiple chromatin remodeling complexes, that FoxP3 might recruit or displace factors

differentially at promoters that are enhanced or repressed by FoxP3. The most

comprehensive way to determine the role of multiple ATP-dependent nucleosome

remodelers and their role in FoxP3-dependent gene regulation would be to utilize a shRNA-

based microarray analysis. Knocking down Mi-2β, Brg-1 or other ATP-dependent

nucleosome remodeling factors and determining the genes differentially expressed in the presence of FoxP3 would provide insight into the remodeling factors required for FoxP3-mediated transcriptional regulation.

The requirement of Mi-2β for Treg function is also unknown. Currently, we are unable to stably knockdown Mi-2β in Jurkat T cells. Regardless, it will be of interest to determine if the knockdown of Mi-2β enhances Treg suppressive activity. Conversely, if Mi-2β is required for general *IL-2* expression, does knockdown of Mi-2β in primary T cells inhibit *IL-2* activation, possibly resulting in anergy induction? Similarly, does the knockdown of Mi-2β lead to a similar inhibition of chromatin accessibility as described in murine Treg cells? Answering these questions will give us a better understanding of the basic mechanisms of gene expression in T cells and mechanisms of FoxP3 regulation in Treg cells.

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