# MOLECULAR MECHANISMS OF *FOXP3*-MEDIATED GENE REGULATION AND THEIR CONTRIBUTION TO T CELL AND THYMIC EPITHELIAL CELL FUNCTION

Virginia K. Hench

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Approved by:

Advisor: Lishan Su

Reader: Jonathan Serody

Reader: Jeffrey A. Frelinger

Reader: Jenny Ting

Reader: Roland Tisch

Reader: Karen P. McKinnon

## ABSTRACT

VIRGINIA K. HENCH: MOLECULAR MECHANISMS OF *FOXP3*-MEDIATED GENE REGULATION AND THEIR CONTRIBUTION TO T CELL AND THYMIC EPITHELIAL CELL FUNCTION

(UNDER THE DIRECTION OF DR. LISHAN SU)

Severe autoinflammatory disease is associated with mutations in the *Foxp3* gene in mice and humans. *Foxp3* is required for the development, function, and maintenance of natural regulatory T cells, a subset of thymic-derived $CD4^{pos}$ T cells that suppress T cell activation and inflammatory processes. Also, Foxp3 expression and function has been reported in thymic epithelial cells (TECs), suggesting a T cell extrinsic function for *Foxp3*[1]. TECs contribute to tolerance and immunity by supporting T cell development.

In chapter two, I will discuss the interaction between FOXP3 and Siva, a previously unknown binding partner of FOXP3. *Siva* is a pro-apoptotic molecule that is expressed across a range of tissues, including $CD4^{pos}FOXP3^{pos}$ T cells. FOXP3 interacts with both the Siva-1 and Siva-2 isoform in 293T cells. The FOXP3-binding domain maps to Siva's C-terminus. A central region of FOXP3 is involved in binding to Siva. *Siva* repressed *IL-2* gene expression in Jurkat T cells. Further, I investigated whether *Siva* and *FOXP3* functionally interact to repress *IL-2* gene expression. The presence of Siva negatively affects the repressive effect of FOXP3 on *IL-2* gene expression. Lastly, we showed that *FOXP3* enhances Jurkat T cell apoptosis in unstimulated cells, but protects from apoptosis in response to stimulation with PMA and Ionomycin. In summary, the data support the hypothesis that both *FOXP3* and *Siva* are negative regulators of T cell activation.

In chapter three, I will present data pertaining to *Foxp3* regulation of *Her2/neu* in TECs and the effect of Her2/neu signaling on thymopoiesis. Previously, it was reported that Her2/neu is upregulated in TECs from *scurfy* mice, a *Foxp3*-deficient strain[1]. We showed that Foxp3 expression was negatively correlated with Her2/neu expression across mouse TEC lines. Further, a Her2/neu-specific monoclonal antibody, Herceptin, partially rescued defective thymopoiesis in *scurfy* mice[1]. To investigate the effect of Her2/neu signaling on thymopoiesis, we treated fetal thymic organ cultures (FTOC) with a dual inhibitor of Her2/neu and EGFR, GW572016 (GW57). GW57 moderately enhanced thymopoiesis in FTOCs, suggesting that GW57 could have therapeutic potential for immunodeficient patients such as HIV-1 infected individuals and those who are recovering from intensive cytotoxic cancer treatments.

*Dedicated to the memory of Charles Joseph Flynn*

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| | |
|---|---|
| 2dG | 2- Deoxyguanosine |
| 7AAD | 7-Aminoactinomycin D |
| AAs | Amino acids |
| AIDS | Acquired immune deficiency syndrome |
| AML-1/RUNX1 | Acute myeloid leukemia 1/runt-related transcription factor 1 |
| AP-1 | Activator protein 1 |
| APC | Antigen prensentation cell |
| APECED | Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy |
| ARRE | Antigen recognition response element |
| AZA | 5-Aza-2'-deoxycytidine |
| BH | Bcl-2 homology |
| BM | Bone marrow |
| CLP | Common lymphoid progenitor |
| CMP | Common myeloid progenitor |
| Co-IP | Co-immunoprecipitation |
| CRAC | $Ca^{2+}$ release activated $Ca^{2+}$ |
| cTECs | Cortical TECs |
| DAG | Diacylglycerol |
| DC | Dendritic cell |
| DD | Death domain |
| DDHR | Death domain homology region |
| DGS | DiGeorge's syndrome |
| DL1 | Delta-like-1 |
| DL4 | Delta-like-4 |
| DMT | DNA methyltransferase |

| | |
|---|---|
| DN | Double negative |
| DP | Double positive |
| E9, E14 | eg., Embryonic day 9, embryonic day 14 |
| EGFP | Enhanced green fluorescent protein |
| EGFR | Epidermal growth factor receptor |
| ER | Endoplasmic reticulum |
| ETP | Early thymic progenitor |
| eYFP | Enhanced yellow fluorescent protein |
| FADD | Fas-associated protein with death domain |
| FGF | fibroblast growth factor |
| Fkh | Forkhead |
| FL | Fetal liver |
| FSC | Forward scatter |
| FTOC | Fetal thymic organ culture |
| GDP | Guanine diphosphate |
| GFP | Green fluorescent protein |
| GITR | Glucocorticoid-induced TNFR-related protein |
| GTP | Guanine triphosphate |
| GW57 | GW572016, small molecule inhibitor of Her2/neu and EGFR |
| HAART | Highly active anti-retroviral therapy |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylase |
| Her2 | Human epidermal growth factor receptor-2 |
| HIV-1 | Human immunodeficiency virus-1 |
| HMT | Histone methyltransferase |
| HSC | Hematopoietic stem cells |
| IBD | inflammatory bowel disease |

| | |
|---|---|
| IKK | IκB kinase |
| IL-2 | Interleukin-2 |
| IL-2R | IL-2 receptor |
| IL2SS | IL-2 start site |
| Ion | Ionomycin |
| IP | Immunoprecipitation |
| $IP_3$ | Inositol triphosphate |
| IPEX | Immunodysregulation, polyendocrinopathy, enteropathy X-linked syndrome |
| ITAM | Immunoreceptor tyrosine based activation motifs |
| $iT_{regs}$ | Induced $T_{reg,}$ peripherally converted $T_{reg}$ |
| KD | Knockdown |
| L.Zip | Leucine zipper |
| LTR | Long terminal repeat |
| LV | Lentivirus |
| M-CSF | Macrophage colony stimulating factor |
| MAPK | Mitogen activated protein kinase |
| MHC | Major histocompatibility complex |
| mTECS | Medullary TECs |
| NFAT | Nuclear factor of activated T cells |
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK | Natural killer |
| NKT | Natural killer T |
| NLS | Nuclear localization signal |
| $nT_{regs}$ | Natural $T_{reg,}$ thymus derived $T_{reg}$ |
| PC | Proprotein convertase |
| PG | pHSPG vector |

| | |
|---|---|
| $PIP_2$ | Phospholipid phosphatidylinositol 4,5 bisphosphate |
| PKC | Protein kinase C |
| PLCγ | Phospholipase C |
| PMA | Phorbol 12-Myristate 13-Acetate |
| PRR | Proline rich region |
| PTK | Protein tyrosine kinase |
| Puro | Puromycin |
| RAG | Recombination activating gene |
| RLU | Relative luciferase unit |
| ROR | RAR-related orphan receptor gamma |
| RV | Retrovirus |
| SAH | Spherical amphipathic helix |
| SCID | Severe combine immune deficiency |
| *Sf* | *scurfy* |
| shRNA | Short hairpin RNA |
| SN | Supernatant |
| SP | Single positive |
| SRBC | Sheep red blood cells |
| SSC | Side scatter |
| STIM | Stromal interaction molecule |
| TAB1 | TAK1-binding protein |
| TAK1 | TGF-β activating kinase |
| TCR | T cell receptor |
| TECs | Thymic epithelial cells |
| TGF-β | Transforming growth factor-β |
| $T_H$ | T helper |
| TIP60 | HIV-Tat interacting protein 60 |

| | |
|---|---|
| TNC | Thymic nurse cell |
| TNFR | Tumor necrosis factor receptor |
| TR1 | Type 1 regulatory |
| TRAF | TNFR associated factor |
| TRAs | Tissue-restricted antigens |
| TREC | TCR excision circle |
| $T_{regs}$ | Regulatory T cells |
| TSA | Trichostatin A |
| TSLP | Thymic stromal lymphopoietin |
| $T_{sup}$ | Suppressor T cell |
| WB | Western blot |
| XIAP | X-linked inhibitor of apoptosis |
| ZnF | Zinc Finger |

# CHAPTER ONE

# BACKGROUND AND INTRODUCTION

## FORWARD

During my time as a graduate student (2003-2010), I studied *Foxp3* function in thymic epithelial cells (TECs) and regulatory T cells ($T_{regs}$), two principal mediators of immune regulation and tolerance. TECs contribute to selection of a diverse T cell repertoire that is poised to fight foreign invaders and is tolerant to self. $T_{regs}$ suppress T cell activation and inflammatory processes.

In this introductory chapter, I will briefly outline aspects of the immune system that provide a cellular context within which *Foxp3* operates. Additionally, I will provide background material that informed investigations and hypotheses discussed in subsequent chapters. First, I will discuss the concept of immune tolerance and cell-mediated tolerance mechanisms. Investigations of the thymus organ, thymopoiesis, TECs, and $T_{regs}$ shaped our understanding of tolerance prior to discovery of *Foxp3.* Although there are some points of deviation, this chapter is loosely organized based on scale, such that explanations of organ and tissue function precede molecular function. Thus, detailed discussion of *Foxp3* does not appear until the second half of this chapter. This chapter concludes with a discussion of Siva, a pro-apoptotic protein, which we identified as a FOXP3-binding partner.

Since its discovery, *Foxp3's* contribution to the $T_{reg}$ phenotype has become well established. In contrast, *Foxp3* expression and function in TECs became controversial during the course of my research. For the sake of clarity, the controversy regarding Foxp3 function in TECs can be found in Chapter Three, along with background information regarding Her2/neu function in TECs.

Finally, in this dissertation, I will adhere to a proposed nomenclature whereby the mouse and human genes are designated as *Foxp3* and *FOXP3,* respectively.

## IMMUNE HOMEOSTASIS THROUGH PROTECTION AND TOLERANCE

### IMMUNITY AND INFLAMMATION

The immune system preserves the integrity of an individual by balancing two endeavors that occasionally operate as opposing forces: defense against infection and tolerance to the organism's own tissues. Additionally, without a healthy immune system, wounds cannot heal. A healthy immune response can clear tumors before malignancy expands, but a chronic unresolved immune response may promote carcinogenesis[2]. Breakdowns in immune regulation mechanisms could be responsible for some aborted pregnancies[3] and for male infertility[4]. Multiple tissues, cell lineages and molecular mechanisms are involved in the immune response process and its regulation[5,6]. Immune pathogenesis ensues when these processes breakdown.

Inflammation is the hallmark feature of an activated immune response. The ancient Romans described inflammation as rubor, tumor, calor and dolor (redness, swelling, heat and pain). In modern times, advanced technologies allow investigation and more precise definition of the molecular and cellular mediators that initiate and restrain inflammation. While inflammation has protective effects, it can also lead to tissue damage. For example, in asthma and inflammatory bowel disease, unresolved inflammation leads to destruction of the airway and bowel epithelium, respectively[7,8]. Generally speaking, acute inflammation is protective and chronic inflammation is deleterious as it is linked to oncogenesis and autoimmune diseases[9].

## TOLERANCE

Tolerance is fundamental to balanced immunity. Conceptually, immune tolerance describes the immune system's ability to discriminate and 'choose' not to form a response. Tolerance is specific and requires a prior encounter with the antigen that one tolerates[10]. Tolerance plays into how the immune system deals with innocuous matter such as non-pathogenic microbes in the gut and the intrinsically non-toxic substances that induce allergic reactions in sensitive individuals[8].

Self-tolerance, the ability to discriminate self from non-self, is one of the most important forms of immune tolerance. Self-tolerance requires the removal and suppression of adaptive immune cells expressing receptors reactive to self. Paul Ehrlich coined the term *horror autotoxicus*[11] to describe a devastating autoimmune Armageddon that was restrained by mechanisms he could not explain. Interestingly, Ehrlich did not believe autoimmune disease to be possible[11].

Unfortunately, autoimmune diseases are possible and have become quite common in developed countries since the middle of the twentieth century[12]. Autoimmune diseases are defined as pathologies resulting from an adaptive immune response against self-antigens[6]. For example, in the nonobese diabetic mouse, an experimental model of type I diabetes, pancreatic T cell infiltration requires islet antigen specificity (Comment in [13]). In contrast, inflammatory bowel disease (IBD) depends on the presence of gut microflora. Although, it is a T cell mediated disease, IBD may not actually depend on self-antigen specific T cells[14].

Conceptually, self-tolerance is simple. It is the process of active immune unresponsiveness towards one's self, which is necessary to preserve health. Figuring out how

tolerance happens is difficult. The early experimental models used to observe tolerance give tangible examples of what tolerance is.

## MEDAWAR'S GRAFT TOLERANCE MODEL

The classic transplantation models used by Billingham, Brent, and Medawar help illustrate how immune tolerance is not passive, but is an active and acquired process[15,16]. Medawar and colleagues performed a series of experiments where CBA mice received either cells or skin grafts from genetically distinct (allogeneic) strain A donors. Adult CBA skin graft recipients rejected skin grafts from A strain donors at around eleven days post-transplant. In contrast, if fetal CBA mice were injected with donor A cell suspensions *in utero*, they accepted the allogeneic skin grafts transplanted eight weeks later. The CBA mice became *tolerant* to the donor A skin graft because they were exposed to donor A antigens very early in life[15,16].

Subsequent experiments performed by Medawar would show that the initial transplant needed to be performed within a time window shortly before or after birth in order to achieve graft tolerance[17]. This same neonatal tolerance window would become relevant when Miller started experimenting with thymectomies[18] and when Sakaguchi's group investigated the relationship between thymectomy and $CD25^{pos}$ $T_{reg}$ production[19].

## CENTRAL AND PERIPHERAL TOLERANCE

Since Medawar's landmark experiments, immunologists have broken down tolerance mechanisms into two categories: central and peripheral. Central tolerance relies on lymphocyte development processes that selectively eliminate cells expressing self-reactive B cell receptors and T cell receptors (TCRs). Unfortunately, central tolerance mechanisms are

not one hundred percent effective. Peripheral tolerance limits immune activation and compensates for self-reactive lymphocytes that escape deletion during central tolerance.

Peripheral tolerance mechanisms include deletion, anergy, and suppression. Anergy is a cell-intrinsic inactivation mechanism that happens after antigen encounter. Anergic T cells cannot proliferate or produce cytokines. Impaired *IL-2* (interleukin-2) expression, in particular, has been used to characterize anergic T cells[20]. Deletion occurs by apoptosis. Cell intrinsic molecular pathways separately regulate anergy and apoptosis, but extracellular triggers can induce both processes. Different T cell subsets derived from within and outside the thymus contribute to peripheral immune suppression[21].

## TOLERANCE AND THE THYMUS

The thymus regulates immune activation and tolerance via the T cells it generates and releases into the periphery. Understanding the importance of thymus function is a relatively recent discovery in the field of immunology that did not happen until the early 1960s[18].

In the last fifty years, the contribution of the thymus to immune tolerance and regulation has been extensively investigated. The thymus selects a diverse pool of T cells that are capable of mounting an immune response to foreign antigen but selectively deletes or suppresses most autoreactive T cells. Because some autoreactive T cells escape from the thymus, peripheral regulation mechanisms are needed to protect the body from immune mediated destruction. One way in which the thymus compensates for generating autoreactive T cells is by generating natural regulatory T cells ($nT_{regs}$), which have dominant immunosuppressive abilities.

## DISCOVERING THYMUS FUNCTION

The thymus is essential for selection of a T cell repertoire that can defend against diverse pathogens and is tolerant to self. However, prior to Jacques Miller's neonatal thymectomy experiments in the 1960s, the common perspective amongst most immunologists was that the thymus was an evolutionary redundancy that served as a lymphocyte graveyard in humans and other mammals[18,22].

The belief that the thymus did not serve a critical function was rooted in observations of adult mice. First, adult thymectomy in mice had no noticeable effect on immune function. Second, while immunologists agreed that immune cells were present in the thymus, the organ lacked germinal centers, which indicated the presence of an immune response in other lymphoid organs. Third, adoptive transfer of thymic lymphocytes did not transfer immune responsiveness to antigen. Finally, the lymphocytes that possessed immune response initiating activity were found in circulation but never entered the thymus. Together, these data were taken to mean that the thymus was unnecessary for immunity[22].

## MILLER'S THYMECTOMY EXPERIMENTS

Miller's thymectomy experiments in newborn mice clearly demonstrated that the thymus was a necessary immune system attribute and laid the foundation for future investigation into thymic function. First, he showed that neonatal thymectomy impaired the immune response against infection and permitted allogeneic skin grafts, which would otherwise be rejected. Next, he showed that thymic grafts could rescue immune function in thymectomized mice. He tested syngeneic and allogeneic thymic grafts. Interestingly, recipients of allogeneic thymus were tolerant to donor histocompatibility antigens, indicating

that some kind of tolerance-inducing selection process might be happening in the thymus. Other groups generated similar data sets around the same time. The significance of the thymus to establishing proper immune function early in life became widely accepted[18].

Thus, Miller's neonatal thymectomy experiments established that the thymus had an immunologic function. The next step was to figure out what the thymus-derived cells did in relation to immune cells from other anatomical compartments.

## Experimental Foundations for B Cell and T Cell Function

The complementary, dual functions of lymphoid cells were first observed in adoptive transfer experiments performed by Henry Claman and colleagues. Claman transferred different combinations of lymphoid organ preparations plus immunostimulatory sheep red blood cells (SRBCs) into irradiated recipients. Antibody production could be assayed by the hemolytic plaque-forming assay. If an *in vivo* antibody response against transferred SRBCs formed, then preparations of splenocytes plus complement would cause plaque formation on sheep blood agar plates[10]. In Claman's system, transferred spleen cells alone generated an antibody response, but neither bone marrow nor thymus alone could. When the bone marrow and thymus preparations were transferred in combination, a robust antibody response was produced. Thus, bone marrow and thymus derived cells synergized to produce an antibody response against the SRBCs. While Claman's system established complementation between bone marrow and thymus derived cells, he did not know which lymphoid source produced the antibody[23].

Next, Miller and his student, Graham Mitchell, used a complex parental/ allogeneic hybrid transfer system to build upon Claman's findings and establish that antibody producing

cells were not thymus derived[24]. At this point, Miller had already shown that thoracic duct preparations shared similar immune reconstitution abilities with thymocytes[10].

To test whether thoracic duct/thymus-derived cells produced antibodies, Miller and Mitchell took adult CBA mice, removed the thymus, irradiated them, and protected them with CBA bone marrow. Two weeks later, they transplanted mice with SRBCs and thoracic duct cells from CBA X C57BL *F*1 hybrids. They waited a week, harvested splenocytes, and selectively depleted different donor cells from the splenocytes by treatment with either α-CBA or α-C57BL sera. If thoracic duct-derived cells from the CBA X C57BL *F*1 hybrids produced antibody, then α-C57BL sera would block plaque formation. The α-C57BL sera did not block plaque formation, but α-CBA did, indicating that the antibody producing cells were derived from the bone marrow and not from thoracic duct cells. In a later experiment, Miller would find that the thoracic duct preparations were mostly comprised of thymocytes[24].

Further experiments from Miller and others established the dual functions of lymphoid cells, whereby bone marrow-derived cells produce antibody and thymus-derived cells enhance antibody production by providing help, hence the term T helper cells ($T_H$)[25]. Eventually, the terms T cell and B cell came to be used to describe thymic-derived and bone marrow-derived lymphocytes, respectively. However, it is worth noting that the term B cell was originally used to describe cells from the bursa of Fabricius, an antibody-cell-producing organ in birds that does not have a known analog in mammals[10,26].

## THE THYMUS AND ADULT IMMUNITY

For many years, the thymus was thought to be dispensable for adult immunity. First, the thymus involutes with age. Involution does not change the gross thymic size. Instead,

adipose tissue expands and takes over the thymopoietic epithelial spaces. In a newborn human, ~95% of the thymus is devoted to thymopoiesis; thymopoietic space comprises only ~10% in seventy year olds. Also, thymectomy, used to remove thymic tumors in myasthenia gravis patients, was not associated with subsequent apparent immunodeficiency[27]. Taken together, these pieces of information were interpreted to mean that ongoing thymic production was not necessary throughout life. The pool of T cells generated early in life would be sufficient to provide immunity and tolerance. Once a diverse pool of T cells was released from the thymus, the organ was no longer needed.

However, Miller had provided data showing that the thymus was needed in adult mice around the same time he published the neonatal thymectomy experiments. He hypothesized that adult mice might require the thymus for immune recovery after irradiation damage. Irradiated, sham thymectomized mice recovered the ability to form an immune response against SRBCs. In contrast, thymectomized mice did not recover from irradiation damage. A similar immune defect was observed in response to skin grafts. Instead of rejecting allogeneic skin grafts, irradiated, thymectomized mice tolerated the grafts many weeks longer than immune-competent controls[28].

These data from thymectomized, irradiated mice were accepted, but most people thought the system was physiologically irrelevant because so many manipulations had been performed. The phenotype Miller observed in his irradiated, thymectomized mice roughly parallels symptoms caused by infection with human immunodeficiency virus-1 (HIV-1). The wasting symptoms and increased susceptibility to infection in AIDS (acquired immunodeficiency syndrome) patients and in the manipulated mice can be attributed to T cell defiency[18].

*The thymus, HIV-1 infection, and aging*

Thymic output studies comparing HIV-1 infected patients to non-infected adults led to a reevaluation of the dogma that the thymus was dispensable to adult immunity. A pivotal study used TCR excision circle (TREC) analysis (see *TREC assays* below) to make two novel observations regarding aging and HIV-1 infection:

1) Thymic T cell production declines with age, but actually continues until age 80, and possibly later[29].

2) HIV-1 infection severely reduces thymic output, but impaired thymus activity can be restored by highly active anti-retroviral therapy (HAART)[29].

Definitive evidence for restored thymic function led to the idea that thymus directed therapies could benefit HIV-1 infected patients, the elderly and patients receiving cytotoxic cancer chemotherapy. These shifts in thinking about the thymus and technological advances have contributed to a growing interest in TEC biology and thymic organ development[30,31].

## THE THYMUS

The main goal of the thymus is to produce a diverse population of T cells that can protect against a broad range of pathogens, but maintain tolerance to self. Although thymocytes are the predominant cell population within the thymus, thymic stromal cells, including TECS, are vital to thymocyte survival and differentiation. Nude mice (spontaneously deficient in *FoxN1*) are deficient in T cells because TECs fail to differentiate and the thymus organ does not form. Even during the earliest stage of organ development, TECs are necessary to recruit hematopoietic precursors to the emerging thymic rudiment.

Crosstalk between thymocytes and TECs continues on during development and persists throughout life; it is necessary for the viability of both populations[32].

The thymus is divided into two main regions: the outer cortex and the inner medulla. The cortico-medullary junction contains blood vessels that allow entrance of thymocyte precursors and exit of mature, naïve T cells. The outer region of the cortex is called the subcapsular region. The early phases of pre-TCR development and positive selection occur in the subcapsular regions and the cortex. Antigen-specific negative selection is thought to occur in the medulla[32,33]. Phagocytic macrophages, dendritic cells (DCs), medullary TECs (mTECs) and cortical TECs (cTECs) are all important stromal cell populations that contribute to thymocyte selection.

## THYMIC ORGANOGENESIS

A basic knowledge of embryonic development is helpful to understanding the earliest steps of thymic formation and organization. Three principal germ cell layers exist in the developing embryo: ectoderm, mesoderm, and endoderm. The endoderm layer divides into two tubes, which form the foundations of the digestive tract and the respiratory system. The pharynx is a chamber space shared in common by both endodermal structures. It contains four morphologically distinguishable pharyngeal pouches[34].

### *Pre-thymic body plan organization during development*

Appropriate development of the thymus requires a network of transcription factors that are expressed within the pharyngeal endoderm prior to thymic rudiment formation. These transcription factors include *Hoxa3, Six1, Eya1, Pax1, Pax9* and *Tbx1*[35,36]. They direct pharyngeal body plan patterning, which is necessary for development of a number of

structures other than the thymus: ear, tonsils, parathyroid, thyroid and lungs[34]. Deletion of any one of these transcription factors results in failure to initiate the thymus and other developmental deformities[35].

*DiGeorge's syndrome and Tbx1*

DiGeorge's syndrome (DGS) is a genetic disease whose etiology is related to defects in embryonic pharyngeal patterning[36,37]. Failure to form a thymus is a defining feature of DGS, but the complexities of DGS go beyond thymic deficiency to include cardiac complications, parathyroid deficiencies, facial anomalies, and problems with speech and hearing. DGS is associated with deletions in chromosome 22 that range in size between 1.5-3 million base pairs. Twenty-four to thirty different genes are encoded by the chromosomal region deleted in DGS[38-40].

Interestingly, one of the earliest gene knockout mice displayed a phenotype very similar to that observed in DGS patients. Like DGS patients, *Hoxa3* (originally called *hox-1.5*) mutants failed to form a thymus or parathyroid, suffered cardiac defects, contained a small thyroid, and exhibited facial abnormalities[41]. The *Hoxa3-/-* creators acknowledged that important chromosomal differences ruled out the possibility that *Hoxa3* would be a DGS candidate; they proposed that the genetic deficiencies causing DGS could be involved in the same development process as *Hoxa3*[41]. Molecular geneticists have since revealed a DGS candidate gene, *Tbx1,* that is involved in pharyngeal patterning, similar to *Hoxa3*[36,42].

*FoxN1*

By comparison to *Tbx1* and the other body plan transcription factors mentioned above, *FoxN1* function is nearly exclusive to the thymus. The skin is the only other organ

besides the thymus known to require *FoxN1*. Hence, the term 'nude' was used early on to describe the hairless athymic mouse phenotype. Immunologists used nude mice as an experimental tool for many years before identifying the *FoxN1* gene[10,43,44].

The primordial thymus originates from the third pharyngeal pouch around embryone day 10 (E10) and can be marked by *FoxN1* expression by E11.25[45]. *FoxN1*-deficient mice arrest thymic organ development at E11.5, because TEC differentiation fails and the thymic anlage can not attract thymocyte precursors[46].

*The 'dual' origin theory of thymus development*

While the emergence of the thymus anlage from the third pharyngeal structure was accepted for the last thirty years, competing theories about the role of the ectodermal layer existed until recently[35]. The 'dual origin' view of thymic epithelial development asserted that the thymic anlage originated from both ectodermal and endodermal layers[6]. A critical attribute of the 'dual origin' theory was that cortical and medullary regions originated from ectoderm and endoderm, respectively[47]. Support for the 'dual origin' theory came from comparative histological studies between wild type and nude mice. Alternatively, the single origin view of thymic development proposed that only the endoderm is necessary and the ectoderm cells are expendable (Reviewed in [35,48]).

*Single endodermal origin of the thymus*

In the 1970s, Le Douarin's chick/quail chimera experiments provided functional evidence that the endoderm was sufficient for thymus formation, the 'single origin' theory of thymic development[49]. The Japanese quail exhibits a unique heterochromatin-staining pattern, which allowed for definitive resolution between quail-derived and chick-derived

cells in chimeras. Following engraftment of the quail endoderm, the chick thymus contained stromal cells from the quail and lymphocytes from the chick[49]. Thus, while the chick/quail chimera studies definitively showed that the entire thymic epithelium emerged from endodermal cells, the 'dual origin' hypothesis was not dismissed until analogous experiments were performed in mice[30,50]. Thirty years later, Gordon *et al*[50] performed *ex vivo* lineage analysis using a cell tracking dye in mouse thymic lobes. They saw no evidence for ectodermal contribution to the thymus organ. Further, E9 ectoderm-free, pharyngeal endoderm cells were sufficient to support thymocyte development when transplanted under the kidney capsules of nude mice[50]. Thus, the entire thymic epithelium is now thought to derive solely from the endodermal layer and this paradigm shift is apparent in the 2008 edition of *Janeway's Immunobiology*[51].

*Bipotent thymic epithelial progenitors*

The endodermal layer contains bipotent TEC progenitor cells (bTEC) that can give rise to both medullary and cortical TECs (mTECs and cTECs, respectively). The presence of bTECs was determined by tracking the fate of single enhanced yellow fluorescent protein (eYFP)-positive endodermal cells in eYFP-negative thymic lobes. $eYFP^{pos}$ cells were present in ~30% of the injected lobes and the eYFP signal colocalized with markers for mature mTECs and cTECs. Thus, single endodermal bTECs have the capacity to form both mTECs and cTECs[52]. Further studies are needed to more precisely define and locate bTECs within total endodermal preparations.

*Thymic mesenchyme*

In addition to endodermal-derived TECs, thymic mesenchymal cells and migratory cells from the neural crest also contribute to thymus formation by releasing epithelial morphogenic factors. Manipulation of the thymic mesenchyme *in vivo* disrupts thymic organogenesis and formation of a thymus organ by re-aggregation requires mesenchymal fibroblasts *in vitro*. The entire set of signals emanating from the mesenchyme during thymic organogenesis is not entirely clear, but one known mechanism is the fibroblast growth factor (FGF) signaling pathway[53]. TECs express the FGF receptor. FGF7 and FGF10, growth factor ligands secreted by thymic mesenchymal cells, promote TEC expansion, even in the absence of mesenchymal tissue[54].

To summarize, multiple factors contribute thymic organogeneis. First, appropriate pharyngeal body patterning must occur. Then, *FoxN1*-dependent TEC differentiation initiates within endodermal-derived tissue. Signals from the thymic mesenchyme contribute to TEC differentiation. Finally, TEC differentiation and maintenance are also dependent upon colonizing thymocyte precursors, which will be discussed in the next section.

## T CELL DEVELOPMENT

*Hematopoiesis*

Early lymphoid cell development occurs in central lymphoid organs, the bone marrow (BM) and the thymus. The liver serves as a central lymphoid organ for the developing fetus. The adaptive immune response develops in peripheral lymphoid organs, which include the spleen and lymph nodes.

The BM is the major site of hematopoiesis in adults and therefore, serves as the main source of new immune cells. Hematopoiesis is the process of blood cell development and differentiation. All blood components including white blood cells, red blood cells and platelets derive from the same progenitors, hematopoietic stem cells (HSCs). HSCs have self-renewing capacity and are vitally significant because they can reconstitute the entire hematopoietic system[55].

The classic HSC differentiation model proposes that the earliest branching step divides the myeloid and lymphoid cell lineages[56]. The common myeloid progenitor (CMP) further differentiates into myeloid-derived immune cells, red blood cells, and platelets. B and T cells differentiate from common lymphoid progenitors (CLPs). B cells complete development in the BM. The thymus organ is required for T cell development, but specialized stromal cells can partially support T cell development *in vitro.*

*OP9 stromal co-cultures and thymopoiesis research*

The OP9-Delta-Like (DL) stromal cell lines have become a powerful tool allowing researchers to dissect mechanisms regulating thymopoiesis. OP9 cells are a stromal cell line derived from the BM of *op/op* mice, which are deficient in macrophage-colony stimulating factor (M-CSF). M-CSF is a myeloid cell differentiation factor that inhibits lymphopoiesis because of its positive effect on myeloid cell differentiation. OP9 stromal cell monolayers support differentiation of B cells and NK cells from embryonic stem cells because they lack M-CSF. Addition of M-CSF to OP9/stem cell co-cultures inhibits lymphopoiesis[57]. However, OP9 cells cannot support T cell development without Notch ligand expression. Constitutive expression of the Notch-1 ligands Delta-like-1 and -4 (DL1/DL4) confer OP9 cells with the

ability to support thymopoiesis[58], because Notch signaling is required for T cell lineage commitment[59]. Although OP9DL1/DL4 cells can support T cell commitment, they cannot support T cell differentiation past the DP stage.

## THYMOPOIESIS

Thymopoiesis, the process of T cell development is also referred to as thymocyte selection. A series of checkpoints selects a diverse pool of T cells that can fight new infections and is mostly devoid of autoreactive cells. The TCR is the principal molecular element that confers a high level of antigen recognition diversity to the adaptive immune system. The conventional TCR is a multi-component complex comprised of variable α/β-subunits and non-variable CD3 subunits. The α/β-chains confer antigen specificity, while the CD3 cytoplasmic tails transmit signaling information, leading to T cell activation[60]. Alternatively, TCRs can form from γδ subunits, leading to formation of γδ T cells. γδ T cells and NKT cells are innate-like effectors that both differentiate in the thymus.

### *ETPs and HSC paradigm revisions*

The differentiation state of the early thymic progenitors (ETPs) that migrate from the bloodstream into the thymus has been an open question for some time. Investigators have asked whether ETPs were similar to CLPs with B cell potential, or, might ETPs have already differentiated towards T-cell lineage commitment?[55] Efforts to define ETPs have led to controversy concerning the first step in the classic HSC paradigm[61]. In contrast to the early myeloid and lymphoid split proposed by the classic HSC model, support for a common T cell/myeloid lineage has come from two separate groups that used complementary assay systems[62,63]. Wada *et al*.[63] performed single cell clonal assays *in vivo* and *in vitro*. Bell and

Bhandoola[62] used the *in vitro* OP9-DL4 differentiation system and identified markers of TCR gene rearrangement within sorted thymic-derived granulocytes. Both groups obtained data showing that ETPs retain myeloid potential, but lack B cell potential. These questions of ETP potential are relevant to understanding the basic biology of the thymus and the etiology of acute myeloid leukemia[56].

*Pre-TCR development*

Once an ETP enters the thymus, it is likely to encounter factors that promote T cell lineage commitment. The pre-TCR phase of development happens in cells described as double negative (DN) because they lack CD4 and CD8 co-receptor expression. During the DN phase, progenitors proliferate, commit to the T-cell lineage and rearrange *TCR* genes. DN thymocyte differentiation phases are defined by CD44 and IL-2 receptor-α (IL-2Rα/CD25) expression. The most immature DN cells are $CD44^{pos}CD25^{neg}$ (DN1) and are not fully committed to the T cell lineage[64].

One example of TEC dependence on thymocytes comes from mice that express the human CD3ε transgene and arrest thymopoiesis at the DN1 stage. As a consequence of DN1 arrest, immature TECs fail to differentiate into mature cTECs[32].

The transition to DN2 ($CD44^{pos}CD25^{pos}$) is driven by thymus-derived signals. cTECs express Notch ligands that are essential for T cell lineage commitment. Delta ligands expressed by cTECs bind to Notch receptors on the thymocyte. Ligation of Notch by Delta-like ligands leads to proteolytic cleavage and release of the Notch intracellular domain (NotchIC), thereby allowing nuclear translocation of NotchIC and transcriptional reprogramming of the thymocyte[64].

Also, cTECS secrete IL-7, which is necessary for thymocyte proliferation and survival. Genetic deletion of *IL-7,* the *IL-7 receptor*, or $\gamma_c$ (an IL-7 receptor component) arrests thymocyte development. Germline *TCRb, TCRg,* and *TCRd* rearrangements initiate during DN2, which prepares thymocytes for the first phase of TCR selection[64].

*TCRβ selection*

Successful *TCRβ* rearrangement and production of a signaling-competent pre-TCR is a necessary checkpoint called β-selection that occurs at the DN3 ($CD44^{neg}CD25^{pos}$) phase. The pre-TCR is a rearranged TCRβ chain paired with the invariant, preTCR-α chain. *TCR* rearrangements depend on RAG (recombination activating genes) enzymes. The *TCR* rearrangement process is stochastic, resulting in 2 out of 3 recombination events that cannot produce a TCRβ protein. Cells that do not make productive *TCR* rearrangements cannot pass the β-selection checkpoint. Once formed, the pre-TCR promotes survival and inhibits apoptosis by cell autonomous signaling[33,65]. Knockout of *RAG* or multiple downstream TCR signaling components (*Lck, Syk, Lat,* and *Slp76*) arrest thymocytes at the DN3 phase[64].

*γδ T cell development*

If *TCRg* and *TCRd* rearrangements make a productive TCR, then thymocytes can become γδ T cells instead of αβ T cells. TCR-ligand interactions appear to be instructive towards the γδ cell fate[33]. γδ T cells share some functions with innate cells, including epidermal maintenance, resistance to certain infections and antigen presentation. Even though γδ T cells form a minor population amongst circulating and lymphoid resident T cells, they comprise 50% of the T cells found at epithelial surfaces[66].

*TREC assays*

The mechanics of *TCR* gene rearrangement leads to the production of excised DNA circles that remain within the cell even after it has egressed from the thymus. Researchers took advantage of a novel PCR-based assay to measure thymic output based on these unused pieces of *TCR* DNA. The intervening pieces of DNA loop out and form circles, called TCR excision circles (TRECs). Because TRECs are extrachromosomal, subsequent cell divisions dilute the presence of TRECs. Therefore, high TREC levels are associated with recent thymic emigrants, while low TREC levels associate with T cells that have undergone multiple rounds of cell division. The TREC assay has been used to show progressive thymic functional decline during aging and in HIV-1 infected patients[29,67].

*DN thymocytes migrate through the cortex*

In addition to proliferating and rearranging *TCR* genes, DN2/3 cells migrate through the cortex away from the cortico-medullary junction towards the outermost subcapsular region in response to chemokine signals. Concentrated TGF-β (transforming growth factor-β) in the subcapsular region may be responsible for inhibiting cell cycle progression and could slow down the transition out of DN3[32].

The *TCRα* loci rearranges following β-selection and the thymocyte transition from DN3 to DN4 ($CD44^{neg}CD25^{neg}$). The DN4 cells upregulate CD4 and CD8 to become double positive (DP, $CD4^{pos}CD8^{pos}$) cells without requiring any identifiable signal[64].

## THYMOCYTE SELECTION AND TCR AFFINITY

The latter phase of thymocyte selection seems to depended upon the affinity of interactions between the TCR and self peptide-MHC ligands presented on thymic stromal cells. Accordingly, if the TCRs interact with self peptide-MHC molecules with moderate affinity, then those cells are positively selected to survive. Conversely, high affinity interactions between TCRs and self-peptide MHC results in either deletion by negative selection or $T_{reg}$ selection, though a bias favors deletion over $T_{reg}$ selection[33,68]. TCRs that do not interact with thymic MHC die by neglect. The thymocyte selection process chooses only ~3% of the initial pool of DP cells to survive[68].

### *NKT cell development*

The NKT cell lineage is an alternative cell fate that can be selected if a DP cell TCR is stimulated by a thymic lymphoid cell, rather than a thymic stromal cell[33]. NKTs share phenotypic features with both NK and T cells. NKTs are considered innate effectors because they can rapidly respond to antigen, leading to production of both pro- and anti-inflammatory cytokines[69].

### *Positive selection and death by neglect*

Positive selection occurs as DP cells migrate through the cortex and interact with cTECs. If the TCRs expressed by DP cells do not interact with self peptide-MHC molecules on cTECs, then those DP cells do not mature and they die from neglect. Host MHC restriction selects T cells in the thymus that will require the same host MHC molecules in the periphery in order to recognize antigen and receive appropriate stimulation cues. TCR

ligation by self peptide-MHC leads to upregulation of the chemokine receptor CCR7, which promotes migration towards medulla, in response to CCL19 and CCL21[32,70].

*Negative selection*

Negative selection is the process by which thymocytes with high affinity for self-peptide-MHC are deleted by apoptosis. It is mediated by BM-derived antigen presenting cells (APCs), including DCs and macrophages, and mTECs. mTECs uniquely contribute to self-tolerance because of their ability to promiscuously express tissue-restricted antigens (TRAs) under control of the autoimmune regulator (*Aire*) transcription factor. *Aire* is essential to T cell tolerance and *Aire* mutations are associated with APECED (derived from autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) disease in humans[71].

**CD4 T CELLS IN THE PERIPHERY**

Upon egress from the thymus, naïve $CD4^{pos}$ T cells have a range of eventual fate options that are dependent upon cytokine signals received in the lymphoid microenvironment during activation. Functionally, conventional $CD4^{pos}$ $T_H$ cells have been associated with facilitating B cell antibody production, enhancing anti-microbial activity in macrophages, recruiting granulocytes, and producing immune suppressive cytokines[72].

In the classic $T_H1$ versus $T_H2$ paradigm, naïve $T_H$ cells have two cell fates available during the course of activation. $T_H1$ cells promote cell-mediated immunity and are needed for intracellular pathogen clearance. $T_H2$ cells promote B-cell antibody production and are needed to remove extracellular parasites. Each cell type is associated with an effector cytokine profile and with specific nuclear transcription factors that commit cells to either the $T_H1$ or the $T_H2$ lineage. Organ specific autoimmune disease was attributed to $T_H1$

perturbations; asthma and allergy were caused by $T_H2$ aberrations. Antagonism between $T_H1$ and $T_H2$ cells has been proposed as a means of immune regulation[72].

The classic $T_H1/T_H2$ dichotomy has been exploded in recent years by the identification of other $T_H$ cell fate options, which will be described in more detail below (*$iT_{regs}$ and $T_H17$ Cells*).

## FROM SUPPRESSOR CELLS TO FOXP3$^{POS}$ $T_{REGS}$

Richard Gershon and Kazunari Kondo first described the existence of suppressor T cells in the early 1970s[73]. Gershon and colleagues observed that combining different thymocyte populations resulted in decreased DNA synthesis compared to one of the populations alone. The experiments were performed in the absence of B cells, so they concluded that a T cell to T cell suppressor mechanism could be at play. They proposed that a putative population of T cells suppressed the proliferative response of other T cells[74]. $T_{regs}$ perform the exact same suppressor function first proposed many years before their identity could be agreed upon[75].

In 1995, Sakaguchi *et al*[76] described a population of $CD4^{pos}CD25^{pos}$ T cells that protected multiple tissues from autoimmune destruction. Depletion of $CD4^{pos}CD25^{pos}$ cells from splenocytes elicited autoimmune disease in multiple organs when transferred into syngeneic athymic nude mice. Reconstitution of $CD4^{pos}CD25^{pos}$-depleted-splenocyte recipients with $CD4^{pos}CD25^{pos}$ cells shortly after transfer protected from autoimmune disease[76]. Thus, the $CD4^{pos}CD25^{pos}$ population contained immune suppressive function that was more ubiquitous than suppressive function associated with any other T cell surface marker combination that had been tested (Reviewed in [73]).

*IPEX patients and scurfy mice*

By 2003, two pieces of evidence established that the *Foxp3* transcription factor inhibited autoimmunity. A naturally occurring X-linked recessive mutation in the *scurfy* mouse was linked to massive lymphocyte accumulation in multiple organs in affected males. Depletion and adoptive transfer experiments established that $CD4^{pos}$ T cells mediated the autoimmune pathology in *scurfy* mice[77]. Second, IPEX (derived from immunodysregulation, polyendocrinopathy, enteropathy X-linked) patients contained mutations in the *FOXP3* gene[78-80].

*FOXP3* AND $T_{REGS}$

The greatest leap forward for $T_{reg}$ biology came in 2003 when two groups linked the development and function of $T_{regs}$ to *Foxp3* in mice. The laboratories of Shimon Sakaguchi and Alexander Rudensky independently performed *in vitro* gene transfer and *in vivo* adoptive transfer experiments to demonstrate that $CD4^{pos}CD25^{pos}$ T cells expressed *Foxp3* and that *Foxp3* expression conferred a suppressive phenotype[81,82]. These experiments suggested that *Foxp3* was necessary and sufficient to confer $T_{reg}$ properties. The $T_{reg}$ functional phenotype is characterized by the ability to suppress effector T cell ($T_{eff}$) cytokine production and proliferation, the inability to produce IL-2 and a hypoproliferative response to antigenic stimulation *in vitro*[73,79]. *Foxp3* expression promotes all aspects of the $T_{reg}$ functional phenotype.

The $T_{reg}$ surface phenotype is associated with constitutive CD25/IL-2Rα expression combined with CD4. However, this surface marker signature is not exclusive to $T_{regs}$, since naïve T cells upregulate CD25/IL-2Rα during activation without acquiring suppressive

properties. Additionally, in newborn mice, the thymus produces $CD4^{pos}CD25^{pos}$ cells that lack *Foxp3* expression and are not capable of suppressing responder cells[83]. Thus, compared to CD25/IL-2Rα, *Foxp3* expression is a more reliable marker of suppressive T cell function.

Recent experiments demonstrated that exogenous *Foxp3* is not always sufficient to confer $T_{reg}$ properties[79]. Feurer *et al*[84] point out that phenotypic data from $Foxp3^{GFP}$ reporter mice and multiple gene array data sets reject the hypothesis that *Foxp3* is a master regulator of the $T_{reg}$ lineage. Other factors upstream of *Foxp3* expression also contribute to the $T_{reg}$ phenotype. Feurer and colleagues proposed that Foxp3 might be thought of as a "*primus iner pares"* or oligarchy member[84]. Still, even if Foxp3 must be demoted, it is still thought to be extremely important for $T_{reg}$ development, function, and peripheral maintenance[84,85].

A wealth of investigations into $Foxp3^{pos}$ $T_{reg}$ biology has revealed a wide range of proposed suppressive mechanisms and target cells. The long list of $T_{reg}$ targets includes T and B lymphocytes, DCs, macrophages, osteoblasts, mast cells, NK cells, and NKT cells. $T_{reg}$ suppression *in vivo* can be contact-dependent and contact-independent. $T_{regs}$ can induce cell cycle arrest and apoptosis by secreting immunosuppressive cytokines (IL-10, TGF-β, and IL-35), consuming immunostimulatory IL-2, inducing cytolysis, and by expressing negative regulators of immune activation on their cell surface[75].

While $T_{regs}$ play a beneficial role in autoimmunity, allergy, and pregnancy, $T_{regs}$ are detrimental in many tumor microenvironments[86,87]. $T_{regs}$ inhibit other immune effectors from attacking and clearing cancerous cells[88,89].

In contrast to the poor prognostic *trans* effects associated with $Foxp3^{pos}$ tumor-infiltrating T cells[88-93], epithelial cell-intrinsic Foxp3 tumor suppressor properties could be

beneficial. Foxp3 functions as a breast cancer tumor suppressor gene that represses the oncogenes *Her2/neu* and *Skp2*[94,95]. More research is needed to dissect the role of *Foxp3* in heterogeneous tumor microenvironments[96].

*Thymic $nT_{regs}$*

Neonatal thymectomy experiments led to the identification of thymic-derived $CD4^{pos}CD25^{pos}$ $T_{regs}$, which have since been designated natural $T_{regs}$ ($nT_{regs}$). $nT_{reg}$ development happens in the thymus and seems to be subject to the same antigen receptor dependent selection processes that are associated with central tolerance and conventional T cell development. $nT_{regs}$ are associated with peripheral tolerance because once they leave the thymus, they are essential contributors to antigen-specific immunosuppression.

The mechanisms regulating $T_{reg}$ selection in the thymus are still unclear, but a few requirements have been identified. High affinity interactions between TCRs and self peptide-MHC class II molecules facilitate $T_{reg}$ selection, but are not sufficient. CD28 co-receptor stimulation and IL-2 are also necessary[97]. Thymic stromal lymphopoietin (TSLP) seems to be involved in $T_{reg}$ differentiation, as recombinant TSLP enhanced *Foxp3* expression in a TSLP receptor-dependent manner in the mouse fetal thymic organ culture (FTOC)[83]. In humans, TSLP production by Hassall's corpuscles has been linked to $T_{reg}$ selection[98]. Finally, $Foxp3^{pos}$ $T_{regs}$ are mostly found within the thymic medulla suggesting that, in most cases, CD4 lineage commitment precedes $T_{reg}$ selection[83,97]. *Aire*-mediated expression of TRAs has been proposed as a means of influencing the $T_{reg}$ TCR repertoire, but thus far, supportive evidence is lacking[99].

Regulatory T cell function is not exclusive to thymic-derived $nT_{regs}$ but dominant immunosuppressive function is closely associated with *Foxp3*. Induced $T_{regs}$ ($iT_{regs}$) that differentiate from naïve T cells outside of the thymus upregulate Foxp3 expression and possess suppressor properties. $iT_{regs}$ control inflammation at mucosal surfaces, such as the lung and the gut[21].

*$iT_{regs}$ and $T_H17$ Cells*

$iT_{regs}$ (also referred to as adaptive $T_{regs}$ by some authors) and $T_H17$ cells are two CD4 T cell subsets, whose regulation seems to be partially dependent upon *Foxp3*[72].

$Foxp3^{pos}$ $iTr_{egs}$ display suppressive properties that are necessary for controlling oral tolerance and inflammation in the gut and lung. Conversion of naïve $CD4^{pos}$ T cells to *Foxp3*-expressing $iT_{regs}$ requires antigen presentation, TGF-β and IL-2. Also, retinoic acid can enhance $iT_{reg}$ expansion *in vitro*. Unlike $nT_{regs}$, *Foxp3* expression is transient in $iT_{regs}$ and depends on continual TGF-β presence[21,100].

$T_H17$ cells are marked by production of IL-17 and have proinflammatory properties that have been linked to organ specific autoimmunity and protection against microbial pathogens. The RORγt (derived from retinoic acid receptor-related orphan receptor γt) transcription factor is needed for $T_H17$ differentiation. Like $iT_{regs}$, $T_H17$ cell differentiation is promoted by TGF-β. However, $T_H17$ production requires the proinflammatory IL-6 cytokine signal to shut down Foxp3 function. In the absence of IL-6 signals, Foxp3 binds and inhibits RORγt function. Foxp3 has a similar effect on RORα-mediated transcription. Thus, the decision to become either an inflammatory $T_H17$ cell or a suppressor $iT_{reg}$ depends on the

balance between RORγt and Foxp3 nuclear transcription factors, which is influenced by cytokines in the extracellular milieu[100].

Foxp3$^{neg}$ $T_{regs}$ have also been described, but the 'lineage' status of these cells is controversial. Type 1 regulatory (TR1) cells exert suppressive effects by producing IL-10 in the gut. Clearly IL-10 is needed to restrain inflammation as *IL10-/-* mice succumb to wasting disease and colitis[101]. However, IL-10 production is associated with multiple T cell subsets, so the IL-10 producing cells identified as TR1 are likely not a unique lineage. Rather, IL-10 production may be a feature shared by different lineages at various times after activation. Also, TGF-β producing $T_H3$ cells were described before identification of the *Foxp3* gene. Reassessment of this population indicates that cells previously designated $T_H3$ cells were probably Foxp3$^{pos}$ i$T_{regs}$[72].

*$T_{regs}$ and apoptosis*

Unique apoptosis response patterns have been observed in $T_{regs}$ compared to CD4$^{pos}$CD25$^{neg}$ cells. However, in some cases $T_{regs}$ have been described as prone to apoptosis and in other cases $T_{regs}$ were relatively apoptosis-resistant. The discrepancies are related to the type of stimulus[102-106], the tissue source of isolated $T_{regs}$[106,107] and the experimental environment (*in vivo* vs *in vitro*)[108,109]. One group reported increased mRNA for anti-apoptotic genes in CD4$^{pos}$CD25$^{pos}$ cells[108], while another group reported that *Foxp3* transgenic CD4$^{pos}$ cells have increased RNA for pro-apoptotic genes[110].

Extensive genomic analyses also have not revealed apoptotic regulators to be major factors associated with *Foxp3* expression or the $T_{reg}$ signature. One exception is gene array data from *Foxp3$^{GFP}$* reporter mice: anti-apoptotic *Bcl-2* was elevated and pro-apoptotic

*caspase-3* was decreased in *Foxp3/GFP*$^{pos}$CD25$^{pos}$ cells compared to *Foxp3/GFP*$^{pos}$CD25$^{neg}$ cells. Related apoptotic regulators did not exhibit a similar expression pattern[111]. Further, genome wide profiling studies of *Foxp3*-dependent genes[112] and genes occupied by Foxp3[113] did not reveal an association between apoptotic genes and *Foxp3* expression or function. Thus, while $T_{regs}$ seem to exhibit apoptotic responses that are often different from conventional CD4 T cells, the underlying mechanism is not clear. The observed differential apoptotic responses seem to be regulated by *Foxp3*-dependent and *Foxp3*-independent factors.

TCR SIGNAL TRANSDUCTION

Investigation of Foxp3 function at the molecular level has been shaped by a long history of research aimed at uncovering mechanisms regulating T cell activation. Full activation of a CD4 T cell requires TCR binding to cognate peptide-MHC class II and stimulation of the CD28 co-receptor. These two signals lead to cytoskeletal rearrangements, proliferation and transcriptional activation within the T cell. T cell anergy occurs when the TCR is stimulated in the absence of CD28 ligation[60].

The conventional αβ TCR is a multi-unit complex expressed on the surface of T cells. The αβ receptor molecules are highly variable and confer antigen recognition specificity to the T cell. The antigen specific αβ pair lacks signal transmission ability, but nonpolymorphic CD3 subunits cluster with the αβ pair and provide signaling capability. The CD3 dimers (γε, δε, ζζ) are dependent upon αβ coexpression[60].

Antigen recognition by the αβ receptor triggers a cascade of phosphorylation events that emanate from the CD3 cytoplasmic tails. The molecular events that translate αβ antigen

binding to CD3 phosphorylation are still unclear. Phosphorylated ITAMs (immunoreceptor tyrosine-based activation motifs) within the CD3 tails recruit PTKs (protein tyrosine kinases) and signaling adaptor molecules that comprise the proximal TCR signaling complex. The multimeric TCR signaling complex activates PLCγ (phospholipase Cγ), which hydrolyzes $PIP_2$ (phosphatidylinositol 4,5-bisphosphate) to chemical messengers DAG (diacylglycerol) and $IP_3$ (inositol triphosphate). DAG activates PKCθ (protein kinase Cθ) and Ras, which leads to AP-1 (activator protein-1) activation[60]. Proximal TCR complex components and PKCθ both contribute to NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation[114]. $IP_3$ activates calcium ion ($Ca^{2+}$) influx, which leads to the activation of NFAT (nuclear factor of activated T cells). The combined presence of AP-1, NFκB, and NFAT in the nucleus robustly activates T cell effector cytokine gene transcription[60].

*Pharmacological TCR simulators*

Many TCR signaling mechanisms have been uncovered in Jurkat cells. Research has been aided by two groups of pharmacological agents: phorbol esters and $Ca^{2+}$ ionophores. Combined application of the phorbol ester, PMA (phorbol 12-myristate 13-acetate), and the ionophore, ionomycin (Ion), simulates TCR stimulation by activating downstream signaling events. PMA activates PKCθ, a target of DAG. Ion raises intracellular $Ca^{2+}$ levels[60].

*TCR downstream signaling*

$Ca^{2+}$ release from intracellular and extracellular sources is triggered by $IP_3$. $IP_3$ receptors cause release of endoplasmic reticulum (ER) $Ca^{2+}$ stores. STIM (stromal interaction molecule) senses ER $Ca^{2+}$ depletion and subsequently opens CRAC ($Ca^{2+}$ release activated $Ca^{2+}$) channels in the plasma membrane, thereby initiating extracellular $Ca^{2+}$ influx. $Ca^{2+}$

binding to calmodulin enables activation of calcineurin. Calcineurin dephosphorylates NFAT, permitting exposure of the NFAT nuclear localization signal (NLS), which directs NFAT to the nucleus[60].

NFAT and AP-1 bind to DNA by forming a cooperative complex. NFAT-mediated gene expression occurs in response to TCR ligation without the need for simultaneous co-receptor binding. In contrast, TCR stimulation only partially enhances AP-1 and NFκB activity. CD28 ligation is needed for optimal activation of the Ras/MAPK (mitogen-activated protein kinase) and IKK (IκB kinase) pathways that will be described next[115].

DAG recruits Ras-GRP (Ras guanyl nucleotide-releasing protein) and PKCθ to the plasma membrane. PKCθ phosphorylates Ras-GRP. Active Ras-GRP replaces GDP (guanosine diphosphate) on inactive Ras with GTP (guanosine triphosphate), yielding activated Ras. Ras then activates Raf, a serine/threonine MAPK enzyme. The MAPK kinase cascade leads to activation of Elk, which is the kinase that activates Fos and Jun. Fos/Jun dimers form the AP-1 transcription factor[60].

The proximal TCR complex activates the canonical NFκB signaling pathway. Genetic studies have shown that multiple TCR-associated PTKs and adaptors are involved. A recently identified PKCθ-dependent trimolecular complex seems also to be important. IKK gets activated by the CARMA, Bcl10, and MALT-1 (CBM) complex [CARMA, caspase recruitment domain (CARD) and membrane-associated guanylate kinase (MAGUK)-containing scaffold protein; Bcl10, B-cell lymphoma/leukemia 10; MALT-1, mucosa-associated lymphoid tissue lymphoma translocation protein 1]. Activated IKK phosphorylates IκB proteins, which leads to their ubiquitin-mediated degradation and release

of sequestered NFκB dimers. Once released, the NFκB subunits translocate from the cytoplasm to the nucleus where they activate gene transcription[114].

*IL-2 signaling and gene regulation*

While multiple cytokines contribute to T cell effector function, IL-2 is essential to immune tolerance and $T_{regs}$, similar to anergic T cells, are characterized by *IL-2* repression. $T_{reg}$ survival requires IL-2[116]. The IL-2 receptor (IL-2R) is composed of three subunits. The IL-2Rα chain (also called CD25) confers high affinity for the IL-2 cytokine. Intermediate affinity for IL-2 is associated with IL-2Rβ. IL-2Rβ binds to IL-2 and IL-15. The third receptor component, $\gamma_c$, has the lowest IL-2 affinity and is essential for signaling by multiple cytokines: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21[117].

For a long time, investigators puzzled over a perceptible paradox regarding IL-2 signaling. IL-2 was required to support T cell activation *in vitro*, suggesting that IL-2 might be necessary to form an immune response. However, *IL-2* deficient animals effectively mounted an immune response against pathogens. Contrary to predictions informed by *in vitro* experiments, *IL-2-/-* animals succumbed to severe autoimmune disease[116,118].

The identification of $T_{regs}$ and their dependence on IL-2 resolves some of the prior confusion regarding IL-2's function *in vivo*. $T_{regs}$ require IL-2 for survival and constitutively express the high affinity IL-2 receptor, but $T_{regs}$ do not produce *IL-2* themselves. Adoptive transfer of wild type $CD4^{pos}CD25^{pos}$ T cells rescued *IL-2Rβ-/-* mice from lethal autoimmunity, indicating that $T_{reg}$ deficiency caused disease in these mice[119].

*IL-2* transcription is a tightly regulated process. $CD4^{pos}$ T cells do not express *IL-2* mRNA in the absence of stimulation, but they quickly turn on *IL-2* transcription once

stimulated[120]. The *IL-2* promoter contains proximal and distal regulatory elements. Approximately 300 base pairs upstream of the *IL-2* start site (*IL2SS*) comprise the minimal, proximal *IL-2* promoter in mice and humans. Mice contain a distal regulatory element, which is responsive to AML-1/RUNX1 (acute myeloid leukemia 1/runt-related transcription factor 1) and is ~1400 base pairs upstream of the *IL2SS*[121]. NFAT, NFκB, AP-1, Oct1 (octamer transcription factor 1) and AML-1/RUNX1 all enhance *IL-2*; Foxp3 represses *IL-2*[117,121].

## FOXP3-MEDIATED GENE TRANSCRIPTION

*Foxp3* is a positive and negative regulator of gene transcription[122]. Extensive genome array analyses have identified hundreds of genes that are both positively and negatively regulated by *Foxp3*[111-113,122,123]. Mouse *Foxp3*-transgenic CD4 cells were resistant to T cell activation, consistent with the observation that many *Foxp3*-dependent genes are involved in T cell activation and anergy[79,113,123]. One of the most well characterized molecular effects of *Foxp3* is *IL-2* repression.

### *Foxp3-mediated IL-2 repression*

Foxp3-mediated *IL-2* repression is associated with direct inhibition of *IL-2*-enhancing transcription factors and by modification of the chromatin structure. Protein to protein binding by co-immunoprecipitation has been shown between Foxp3 and transcriptional co-activators that bind to the *IL-2* proximal promoter: NFκB (p65), NFAT[124,125], and AP-1 (c-Jun)[126].

Foxp3 represses *IL-2* by binding NFAT and inhibiting NFAT:AP-1-dependent activity. In the absence of Foxp3, the NFAT:AP-1 complex activates *IL-2* at the ARRE (antigen recognition response element), a composite binding site for NFAT:AP-1 complexes.

Foxp3 inhibited NFAT's ability to activate an ARRE reporter and required NFAT in order to bind the ARRE DNA sequence[125].

*Foxp3 and chromatin modifications*

*Foxp3* also regulates gene transcription by recruiting a complex of chromatin modifying enzymes and modulating histone acetylation in a promoter sequence specific manner[127-130]. Chromatin consists of DNA tightly wrapped around histone proteins. Eight histone units and 146 DNA base pairs comprise a single unit of chromatin[131]. Histone acetyl modifications maintain chromatin in an open, transcriptionally active state. The histone code hypothesis predicts that inactive gene promoters will be associated with reduced histone acetylation and inaccessible chromatin[132]. Foxp3 decreases *IL-2* promoter acetylation and represses *IL-2* gene transcription, which is consistent with the histone code hypothesis[129,133].

A number of enzyme groups regulate chromatin structure by the addition or removal of acetyl and methyl modifications. Histone deacetylase (HDAC) enzymes remove acetyl groups leading to condensed, transcriptionally inactive chromatin[131]. HDAC activity seems to promote DNA methylation by DNA methyltransferases (DMTs), but the enzymatic activities have yet to be definitively linked at the molecular level[134]. DNA methylation is associated with transcriptionally inactive chromatin[135]. Chromatin modifications are reversible. Histone acetyltransferase (HAT) enzymes 'open' up chromatin structure by adding acetyl groups on to histones. In addition, certain histone methylation marks are specific to either open or closed chromatin structure. Histone methyl transferases (HMTs) are distinct from DMTs. The importance of chromatin structure to transcriptional regulation is highlighted by the

observation that HATs, HDACs, and HMTs have been found 'traveling with' RNA polymerase II[131].

A number of lines of evidence support the hypothesis that *Foxp3* modulates gene expression by affecting chromatin structure. First, ectopic *Foxp3* induces promoter specific alterations in chromatin structure in a manner that is consistent with the histone code hypothesis. Exogenous *Foxp3* repressed *IL-2* and reduced histone acetylation at the *IL-2* promoter[130,131]. Increased histone acetylation was observed at the promoters of genes that are upregulated by *Foxp3* overexpression in CD4 T cells (*GITR, CTLA-4* and *CD25*)[125,129]. Second, Foxp3 co-immunoprecipitated with HDAC7, HDAC9, and the HAT, TIP60 (HIV-Tat interacting protein 60). TIP60 enhancement of Foxp3-mediated transcriptional repression was dependent upon HAT enzyme function. Maximal repression of *IL-2*-transcriptional activity required *Foxp3, TIP60,* and *HDAC7*[128]. Third, Zheng *et al*[122] reported that many chromatin remodeling genes showed up in their genome-wide analysis of *Foxp3* target genes. Fourth, another recently identified Foxp3-binding partner, Eos, is also associated with chromatin remodeling. Eos was required for repression of *IL-2* and silencing of Eos in $T_{regs}$ blocked repressive histone modifications within the *IL-2* promoter[136]. Finally, Eos, TIP60, and HDAC7 bind to Foxp3's transcriptional repressor domain[128,136].

## FOXP3 DOMAIN FUNCTIONS

The mechanisms by which Foxp3 mediates its effects have been informed by characterizing Foxp3 domains that are required for or associated with specific molecular functions. The defined putative structural domains of full length Foxp3 (mouse, amino acids (AAs) 1-429, Figure 2.1) include the N-terminal proline rich region (PRR), the zinc finger

(ZnF), the leucine zipper (L.Zip), and the forkhead (Fkh) domain. Extensive investigations into Foxp3 function have uncovered specific activities associated with particular regions.

Foxp3 function and nuclear translocation are partially mediated by proteolytic cleavage at two proprotein convertase (PC) motifs located near the N- and C-termini. *In vitro* and *in vivo* assays revealed that *Foxp3* PC motif mutations altered $T_{reg}$ suppressive function. The first PC motif is located within Foxp3's first exon, between AAs 48-51; the latter is near the end of the C-terminus (AAs 414-417)[137,138].

Truncation analysis by Steven Ziegler's group identified two transcriptional repressor regions within the Foxp3 N-terminus. The first repressor domain spans AA's 67-132 and overlaps with the exon 2-encoded domain (AAs 72-105)[79,139]. The second repressor domain (AAs 135-198) is required for NFAT-mediated transcriptional repression[79].

The region encoded by *Foxp3's* second exon (AAs 72-105) binds and inhibits the transcriptional activities of RORα and RORγt[140,141] A FOXP3 isoform that lacks exon 2 was identified in humans, FOXP3Δ2[142].

Histone modifying factors associate with both Foxp3 repressor regions (AAs 67-132, AAs 135-198)[79]. AAs 106-190 are required for binding to HDAC7 and TIP60[128]; AAs 148-198 bind Eos, a transcription factor associated with histone modifications in the *IL-2* promoter[136].

AAs 199-223 encode the Foxp3 ZnF domain. No known function is associated with the ZnF and it is the only described domain that is not associated with a sequenced IPEX mutation[139].

The L.Zip domain (241-268) is required for Foxp3 homodimerization and $T_{reg}$ suppressive function[139,143,144]. The IPEX Δ250 mutation is sufficient to block suppressor function *in vivo* and *in vitro*[143]. The IPEX mutation Δ251 blocks *FOXP3*-mediated *IL-2* inhibition[144].

The region between the Foxp3 L.ZIP and the Fkh domain (AAs 278-337) contains AML-1/RUNX1 binding activity. Triple point mutations at AA residues 329/330/332 inhibit Foxp3:AML-1/RUNX1 binding, block IL-2 repression, and suppressor cell function[121].

The Fkh DNA binding domain is highly conserved amongst all members of the forkhead box (Fox) family[145]. The Foxp3 Fkh domain is required for DNA binding, nuclear translocation, cytokine transcription regulation, and NFAT-mediated suppression[125,139]. The Fkh domain contains more IPEX mutations than any other single domain. Our group has shown that the Fkh domain is required for NFκB-mediated HIV-1 LTR (long termninal repeat, HIV-1 sequence containing promoter activity) enhancement[130].

## SIVA: PRO-APOPTOTIC MOLECULE WITH T CELL SIGNALING PROPERTIES

Our efforts to understand FOXP3 function on the molecular level led to identification of a previously unknown FOXP3-binding partner, Siva. Siva is a pro-apoptotic molecule that is also involved in T cell signaling.

Siva was originally identified in a screen for CD27 binding partners and was named after the Hindu god of destruction[146]. Subsequent investigations have revealed multiple Siva binding partners that are associated with diverse cellular functions (shown in Table 1.1). In addition to binding CD27, Siva interacts with two other members of the tumor necrosis factor receptor (TNFR) family, GITR (glucocorticoid-induced TNFR-related protein) and OX40[147].

Siva has been localized to multiple subcellular compartments (Table 1.2). Using a panel of mouse and human tissues, investigators detected abundant Siva mRNA in most tissues screened by Northern blot[146,148]. Two isoforms, Siva-1 and Siva-2, are present in mouse and human[148,149].

*Siva and T cells*

Although many reports focus on Siva's pro-apoptotic function, Siva has been described as a regulator of T cell signaling. Siva inhibits NFκB activity by blocking IκBα degradation, leading to reduced levels of nuclear NFκB[150]. Another group found that Siva associates with TAK1 and TAB1 (TAK1, TGF-β activating kinase and TAB1, TAK1-binding protein), signaling adaptors that activate both NFκB and AP-1. XIAP (X-linked inhibitor of apoptosis) inhibited Siva's association with TAK1 and TAB1 and promoted Siva-mediated AP-1 activation[151].

Siva binds to the cytoplasmic tail of CD27, a member of the TNFR superfamily. TNFRs are transmembrane receptors that mediate cell death and survival by binding to death-domain (DD) and TRAF (TNF receptor associated factor) adaptors, respectively. DD-molecules activate apoptosis and TRAFs are generally associated with NFκB- and AP-1-mediated survival signals[152]. However, some TNFRs, including CD27, do not contain DDs in their cytoplasmic tails. Thus, Siva provides a means by which CD27 has the capacity to transmit a pro-apoptotic signal. CD27 promotes survival by activating NFκB and c-Jun via TRAF-2[153].

CD27 expression is associated with naïve, effector and memory T cells, but is elevated during the effector phase. *In vitro,* CD27 supports T cell clonal expansion.

Systemically, CD27 is associated with formation of anti-viral immunity. *CD27* knockout mice showed reduced antigen-specific naïve T cell expansion and impaired $CD8^{pos}$ T cell memory in response to influenza virus[153,154].

CD27's ligand is CD70, a member of the TNF family. Structurally, CD70 is related to TNFα, but unlike TNFα, CD70 remains anchored to the plasma membrane. CD70 is expressed by lamina propria APCs, and activated T and B cells[146,155]. CD70 transgenic mice (CD70), whose B cells constitutively expressed CD70, exhibited immune hyperactivation that resembled HIV-1-related immunodeficiency. Naïve T cells were depleted in CD70Tg mice due to excessive effector-memory cell conversion. Around age 6-8 months, CD70Tg mice died from *Pneumocystis carinii* infection, a characteristic of T cell deficiency[156]. Further support for CD70-mediated immune activation came from an adoptive transfer model of IBD. CD70 blockade suppressed IBD[155]. In contrast to CD70's T cell activating role in the CD70Tg mice, CD70 on kidney carcinoma cells promoted lymphocyte apoptosis, which was associated with Siva expression[157].

*Siva protein domain organization*

The authors who first identified Siva defined three domains based on sequence homology to other known protein functional domains (Figure 2.1A)[146]. The amino terminus was not classified. The central region was named the DD homology region (DDHR) for its similarity to DDs in FADD (Fas-associated protein with death domain) and RIP (receptor interacting protein kinase 1). Siva-2 lacks the second exon, which includes most of the DDHR[148]. The Siva C-terminus is enriched with cysteine residues. The authors designated a putative B-box and a putative ZnF domain within the Siva C-terminus[146]. Further, the Siva

primary sequence predicts formation of a spherical amphipathic helix (SAH), a tertiary structure that segregates hydrophilic and hydrophobic residues to either side of the helix[158]. Specific molecular functions have been associated with all known Siva domains (See Table 1.3).

*Siva and apoptosis*

Activation of mitochondrial-dependent apoptosis by Siva has been described in T lymphocyte[159] and breast cancer cell lines[158,160-162], but the mechanisms by which Siva induces apoptosis may be cell type-dependent. In breast cancer cells, Siva's SAH domain binds Bcl-2 and Bcl-$X_L$ and inhibits their protective effects[158,160-162]. However, the full SAH domain is not required to induce apoptosis in T lymphocytes[159]. The SAH domain spans the exon-1/exon-2 junction. Exon-2 is missing from Siva-2, yet Siva-1 and Siva-2 induced equivalent levels of caspase-3 activity in T cells. One explanation could be that the exon-1 portion of the SAH is sufficient to induce caspase-3 activation. However, the Siva C-terminus, which completely lacks the SAH domain, induced equivalent levels of phosphatidyl serine exposure compared to the Siva N-terminus. Thus, in contrast to breast cancer cells where the SAH domain is sufficient to induce apoptosis, more than one domain of Siva contributes to apoptosis in T cells. Further, since phosphatidyl serine exposure and caspase-3 activation are downstream of both the mitochondrial-dependent and -independent apoptotic pathways, the Siva truncation experiments leave open the possibility that Siva could activate mitochondrial-independent apoptosis in T cells[159]. More investigations are needed to clarify whether Siva does indeed use more than one mechanism to induce apoptosis in different cell types.

| Siva Binding Partner | Related Cellular Functions and Comments |
|---|---|
| CD27 | The CD27 cytoplasmic tail binds Siva-1 and Siva-2[146,148]<br><br>Ligation of CD27 by CD70 promotes apoptosis in the presence of Siva overexpression[146,159].<br><br>Binding between CD27 and Siva could NOT be detected in 2 myeloma cell lines transfected with Siva or in a $CD27^{pos}$ B cell lymphoma line[79] |
| CVB3 VP2 protein | Mediates apoptotic response to Coxsackievirus infection in myocardial cells[163,164] |
| ARG Tyrosine Kinase | ARG phosphorylation of Tyr-34 in Siva-1 &-2 mediates apoptosis in response to oxidative stress[165] |
| GITR, OX40 | Siva-1 binds both and functionally interacts with GITR to promote apoptosis[147] |
| BCL-$X_L$, BCL-2 | Siva promotes apoptosis by binding and inhibiting BCL-$X_L$ and BCL-2. Siva does not bind Bax, a pro-apoptotic member of the BCL-2 family[158,162] |
| Pmp22 | Peroxisomal membrane proteins regulate fatty acid and hydroperoxide metabolism. The functional interaction between Siva and Pmp22 is undefined[166] |
| HPV-16 E7 Protein | HPV-16 E7 competitively blocks Siva binding to BCL-$X_L$ and blocks the pro-apoptotic function of Siva in response to UV radiation[167] |
| CD4 | Siva enhanced HIV-1 envelope-induced apoptosis[168] |
| Pyrin | Pyrin blocks the pro-apoptotic effect of Siva in response to oxidative stress[149] |
| $LPA_2$, $S1P_4$ | $LPA_2$ degrades Siva in the presence of growth factors[169,170] |
| SLIMMER | SLIMMER, a muscle protein, inhibits Siva-mediated apoptosis[171] |
| Telethonin | Like SLIMMER, Telethonin is a muscle cytoskeletal protein that localizes to sarcomere Z-discs. The functional interaction between Siva and telethonin is undefined[172]. |
| XIAP | Anti-apoptotic XIAP inhibited Siva by proteasomal degradation[151] |

**Table 1.1: Siva interacting partners and associated functions**

| Siva Sub-Cellular Localization | Cell Line or Tissue & Comment |
|---|---|
| Plasma membrane association during neuronal apoptosis for endogenous Siva | γ-irradiated whole brain was submitted to subcellular fractionation[173] |
| Cytoplasmic & Mitochondrial | In transfected HeLa cells, Siva co-localized with mitotracker[158] |
| Translocation to the mitochondria in response to DNA damage agent | HCT116 colorectal carcinoma cells were treated with cisplatin[160] |
| Predominantly nuclear in the absence of $LPA_2$<br>The presence of $LPA_2$ or $SIP_4$ recruited Siva to the cytosol and plasma membrane | NIH3T3 fibroblasts were grown in serum free condidtions[169,170] |
| Cytoplasmic & nuclear in undifferentiated myoblasts<br>Cytoplasmic in differentiated myotubes<br>Z-line localization in mature muscle cells | Skeletal muscle cells[171] |
| Nuclear colocalization with p53 | H1299 lung cancer cells and A549 pulmonary epithelial cells[174] |
| Nuclear (diffuse staining becoming punctate by 24-hours post transfection) | Human Peripheral Blood Acute Lympohcytic Leukemia (HPB-ALL) T cells[159] |
| Siva-1 formed distinct nuclear aggregates in the presence of Pyrin<br>Siva-2 displayed nuclear and cytoplasmic distribution in the presence of Pyrin | Cos-7 cells[149] |

**Table 1.2: Siva sub-cellular localization**

| Siva Domain | AA Position | Function |
|---|---|---|
| N-terminus | 1-47 | Is sufficient to induce apoptosis in HPB-ALL T cells; Mediates nuclear translocation[159]<br><br>Needed for binding to anti-apoptotic HPV-E16 protein[167]<br><br>Binds to CD27 and likely mediates binding to GITR and OX40, since these three TNFRs share a homologous motif associated with Siva binding[147,166]<br><br>Binds peroxisomal membrane protein-22 (PMP22)[166] |
| N-terminal Tyrosine | Tyr-34 | Mediates apoptotic response to oxidative stress in collaboration with ARG tyrosine kinase[165] |
| SAH | 36-55 | Necessary and sufficient to promote apoptosis in breast cancer cell lines by binding and inhibiting BCL-$X_L$ & BCL-2[158,161,162] |
| Exon 2 Domain | 40-103 | Mediates homooligimerization of Siva-1, which may be required for Siva-1 to inhibit p53 stability[174]<br><br>Inhibits binding to Pyrin[149]<br><br>Binds 1 zinc ion[166] |
| DDHR | 48-121 | Is NOT sufficient to induce apoptosis in HPB-ALL T cells[159]<br><br>Binds the VP2 protein from the virulent Coxsackievirus B3 strain (AAs 114-122)[164] |
| C-terminus | 114-175 | Is sufficient to induce apoptosis in HPB-ALL T cells[159]<br><br>Binds 2 zinc Ions[166]<br><br>Binds LPA Receptor-2 cytoplasmic tail[169]<br>Binds CD4 cytoplasmic tail[168]<br>Binds muscle sarcomere Telethonin[172]<br><br>Dispensable for binding XIAP[151] |

**Table 1.3: Siva functional domains**

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# CHAPTER TWO

# REGULATION OF T CELL ACTIVATION BY *SIVA* AND *FOXP3*

ABSTRACT

Severe autoinflammatory diseases are associated with mutations in the *Foxp3* locus in both mice and humans. *Foxp3* is required for the development, function, and maintenance of regulatory T cells ($T_{regs}$), a subset of CD4 cells that suppress T cell activation and inflammatory processes. Here we show an interaction between *FOXP3* and *Siva*, a previously unknown *FOXP3* binding partner. *Siva* is a pro-apoptotic molecule that is expressed across a range of tissues, including $CD4^{pos}FOXP3^{pos}$ T cells. We have mapped the FOXP3-binding domain to Siva's C-terminus. A central region of FOXP3 contains Siva-binding activity. We show that *Siva* represses IL-2, a cytokine involved in T cell activation and critical to immune tolerance. We observed that the repressive effect of FOXP3 on *IL-2* transcriptional activity is enhanced by *Siva* knockdown and diminished by *Siva* overexpression. Siva's repressive effect on *IL-2* expression appears to be mediated by inhibition of NFκB. Siva overexpression did not affect NFAT or AP-1 transactivation potential in activated cells. Finally, we showed that *FOXP3* moderately enhanced Jurkat T cell apoptosis in unstimulated cells, but protected Jurkat T cells from apoptosis induced by stimulation with PMA and Ionomycin. In summary, the data support the assertion that both *FOXP3* and *Siva* function as negative regulators of T cell activation.

## INTRODUCTION

The transcription factor Foxp3 is essential for immune system regulation due to its role in the development and function of regulatory T cells ($T_{regs}$)[1,2]. The dramatic autoimmune phenotype in both mice and humans caused by mutated *Foxp3* led to its initial identification[2,3]. In the absence of Foxp3, lethal autoimmunity ensues. Sequencing the *FOXP3* genes from IPEX (Immune polyendocrinopathy, enteropathy, X-linked) patients revealed function ablating mutations throughout domains critical for FOXP3 function[4,5]. The *scurfy* mouse is an autoimmune mutant that has a spontaneous truncation mutation in *Foxp3*. In addition to its well-studied role in $T_{regs}$, an emerging body of work has revealed Foxp3 to be a tumor suppressor in breast cancer[6,7]. Foxp3 activates and suppresses a broad range of genes, but the mechanisms by which this happens are not well understood[8-10]. By understanding the relationship between FOXP3 and its binding partners, we hope to illuminate how FOXP3 operates as a powerful regulator of immune activation.

Already, Foxp3 is reported to reside in a supramolecular complex[11] and a number of specific interactions have been identified[12]. Co-immunoprecipitation (Co-IP) demonstrated binding between Foxp3 and the following partners: NFκB p65[13], TIP60, HDAC7, HDAC9[14], FoxP1[11], AML-1/RUNX1[15], AP-1 family member, cJUN[16], RORα[17], RORγt[18,19], and Eos[20]. Also, Foxp3 homo-oligomerizes[11,21]. Wu *el al*[22] demonstrated that Foxp3 inhibits the transcriptional enhancing effects of NFAT and AP-1 by disrupting their interaction.

Regulation of *IL-2* gene expression is critical to immune tolerance, $T_{reg}$ development and function[23,24]. Foxp3 inhibits *IL-2* production in $T_{regs}$ and confers *trans IL-2* suppressive function[25]. Even before $T_{regs}$ and *Foxp3* became inextricably coupled, researchers

investigated the effect of Foxp3 on *IL-2* transcription[26]. While *IL-2* is not the sole target of Foxp3[9], coordinated investigations into molecular interactions localized to the *IL-2* promoter have been a successful strategy thus far, towards understanding Foxp3's function as a transcriptional regulator.

Here we report a previously unidentified FOXP3 binding partner, Siva. The novel interaction was exposed in a yeast two-hybrid screen for FOXP3 binding partners. We were interested in Siva for its known cell death promoting properties[27,28]. The possibility of a pro-apoptotic molecule that might confer $T_{reg}$ properties was intriguing. Also, Siva binds tumor necrosis factor receptor (TNFR) family members associated with (but not exclusive to) the $T_{reg}$ surface phenotype: GITR (glucocorticoid-induced TNFR-related protein), CD27, and OX40[29-33]. GITR is highly expressed on $T_{regs}$ and attributes suppressive properties under certain conditions[34,35]. Siva and GITR functionally interacted to exacerbate apoptosis in a transient transfection system[29]. Thus, we investigated Siva because of its pro-apoptotic properties and its ability to bind TNFR family members that are associated with the $T_{reg}$ surface phenotype.

Our data shows a physical interaction between FOXP3 and Siva protein expressed in 293T cells. We mapped the FOXP3-interacting domain to the C-terminus of Siva. The central portion of FOXP3 (amino acids (AAs) 106-332) contains Siva-binding activity. We found that Siva repressed IL-2. Additionally, based on *Siva* knockdown (KD) and overexpression experiments, we observed that the relative repressive effect of FOXP3 on *IL2* gene expression was reduced by the presence of Siva. Therefore, *Siva* and *FOXP3* could functionally interact to repress *IL-2* gene transcription, but further investigations are needed to determine whether the biophysical interaction and the functional interaction are

molecularly linked. The repressive effect of Siva on *IL-2* appears to be mediated by NFκB, as our data and others show a negative regulatory effect for Siva on NFκB activity[36]. In summary, while the pro-apoptotic effect of Siva has been thoroughly demonstrated and documented, this analysis of *Siva's* effect on *IL-2* contributes evidence for the protein's role in T cell signaling.

## METHODS AND MATERIALS

### *Cell culture and plasmids*

293T cells were grown in DMEM-10% FetalPlex (Gemini). Jurkat cells were grown in RPMI 1640-10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. When needed, Jurkat cells were selected in 1 μg/ml puromycin (puro). Puro selection took 3-10 days, depending on transduction efficiency. Puro selection was judged to be complete once cellular debris events measured by Gauva EasyCyte reached less than 10%. Unless otherwise stated, all tissue culture reagents were purchased from Invitrogen.

Myc/FOXP3 full length and all derivative truncation mutants were expressed in pCDNA3.1 and made as previously described[10].

The human *Siva-1* cDNA was purchased from ATCC. By PCR subcloning, we transferred *Siva-1* from pCMV-SPORT6 into pEGFP-C1 to generate an enhanced green fluorescent protein(EGFP)/Siva-1 fusion construct. All Siva truncation mutants were derived from pEGFP/Siva-1 by PCR subcloning. Splice overlap extension was used to generate Siva-2 and ΔBbox mutants[37]. *Siva-1* was subcloned into the retroviral vector pHSPG under control of the MSCV promoter[38].

A panel of five pLKO lentiviral (LV) vectors expressing short hairpin targets against Siva was purchased and tested for knockdown efficiency (Open Biosystems). Targets were named based on the last 3 digits of the manufacturer's clone ID. pLKO contains a puro selection cassette and the U6 promoter controls hairpin expression. pLKO-shEGFP and pLL5.0-NS served as a negative controls in designated experiments. The IL-2-luciferase and

NFAT-luciferase reporter plasmids were gifts from Dr. Gerald Crabtree. Dr. Albert Baldwin provided pMHC-3x-κB-Luc.

*Yeast two hybrid screen*

A human thymus cDNA library was screened for FOXP3 binding partners. The bait plasmid contained the full length *FOXP3* sequence in-frame with the Gal4 DNA binding domain (BD). The prey plasmids contained cDNA clones from the human thymus library adjacent to the Gal4 activation domain (AD). cDNA clones were amplified using random primers and oligo dT primers to generate partial and full-length cDNA clones, respectively. Interaction between the DNA-BD and AD permits transcription of reporters for histidine (*His),* adenine (*Ade)*, and β-galactosidase (β-gal) synthesis. *His-/Ade-* dropout media and blue/white screening for β-gal activity permitted selection of clones containing FOXP3 interacting partners. Another level of selection criteria was provided by increasing doses of the *His* inhibitor, 3AT.

*293T transfections, co-IPs, and antibodies*

293T cells were co-transfected with Effectene according to the manufacturer's protocol (Qiagen). 4 μg total plasmid DNA was added to sub-confluent cells in 10-cm dishes. At 48-hours post-transfection, lysates were harvested in PBS, washed once and stored at -20°. Cell pellets were lysed in modified RIPA (Tris-HCl, pH 7.35, 50 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40 1%; NaDeoxycholate 0.25%) supplemented with Complete EDTA-free Protease Inhibitor (PI, Roche), PMSF (100 mM), and Sodium Flouride (NaF, 50 mM). Immunoprecipitations (IPs) were performed in ~1 ml total volume PBS (supplemented with PI, PMSF and NaF): 500 μg protein lysate and 40 μl mouse anti-Myc 9E10 hybridoma

supernatant (kindly provided by Dr. Yue Xiong) were added to PBS. The protein lysate comprised 5-10% of the IP volume. IPs were incubated overnight at 4° on a rotator; Inputs were mixed with SDS loading dye and stored at 4°. A 1:1 mixture of Immobilized Protein A (Invitrogen) and Protein G Agarose (ThermoScientific) beads was prepared and washed three times with PBS. 40 µl of bead mixture were added to each IP. IPs with beads were incubated 2-4 hours at 4° on a rotator. Beads were washed four times with cold PBS (no supplements). Washed beads were resuspended in 20 µl SDS loading dye and heated to boiling in a 95° sand bath (~10 minutes). Boiled samples were run on 10% NuPage minigels according to the manufacturer's instructions (Invitrogen). Following transfer, PVDF membranes were blocked with PBS-Tween 0.2%, 5% milk, which was used for all subsequent steps. Membranes were incubated with mouse α-GFP (Clontech, Living Colors A.v. Monoclonal Antibody (JL-8), 100 ng/ml) to detect immunoprecitation of EGFP/Siva fusion constructs. Membranes were stripped and reprobed with rabbit α-Myc sera (Also, provided by Dr. Yue Xiong).

*Primary cell culture and stimulation*

We isolated $CD4^{pos}$T cells from PBMCs by negative selection with magnetic beads. Subsequently, we sorted $CD4^{pos}$ T cells by positive selection for CD25 expression. Immediately following isolation, half of the freshly isolated cells were washed and set aside for mRNA isolation. The other cells were treated with anti-CD3 and anti-CD28 for 24-hours, prior to colleting pellets and storing them in Qiagen RLT buffer at -80° in preparation for mRNA isolation.

*Standard RT-PCR and quantitative PCR*

The Qiagen RNeasy isolation kit was used to isolate mRNA from frozen cell pellets preserved at -80° in Qiagen RLT buffer containing β-mercaptoethanol. Reverse transcription reactions were performed with random primers and M-MLV reverse transcriptase in the presence of RNase inhibitor. Quantitative Taq-Man PCR was performed using primers from Applied Biosystesms. The following primer sequences were used in semi-quantitative RT PCRs:

*Siva For,* 5'- TACAGCTCAAGGTCCGCGTGAGC-3',

*Siva Rev,* 5'-TCACTGCAGTCCACGAGGCCACA-3',

*β-actin For,* 5'- TCACCCACACTGTGCCCATCTACGA-3',

*β-actin Rev,* 5'- CAGCGGAACCGCTCATTGCCAATGG-3'

*BioGPS data analysis*

Siva expression data for mouse T cell subsets was obtained from the GeneAtlas MOE430, gcrma[39] micro-array profile through the public access gene portal, BioGPS[40]. Arrays were probed with RNA isolated from a broad spectrum of tissues and cell lineages. Four reporters are associated with the mouse *Siva* gene sequence. The 1433680_x_at and 1418377_a_at reporters were excluded because of extremely weak spot intensity compared to the 1452020_a_at and 1426323_x_at reporters. Spot intensity values were normalized against $CD4^{pos}$ T cells so that data from spots with different re-activities could be subjected to statistical analysis.

*Immunofluorescence*

U2OS were plated onto chamber slides one day before being transfected with plasmids expressing Myc/FOXP3 and pEGFP/Siva-1, or appropriate vector controls. At 48-hours post-transfection, the media was removed. Cells were fixed and stained with either Rhodamine conjugated-IgG isotype or Rhodamine anti-Myc.

*Retrovirus production and transduction*

The calcium phosphate method was used to transfect 293T cells with pHSPG, VSV-G, and gag/pol plasmids[41]. Retroviral (RV) supernatants (SN) were collected at 48 and 72 hours and titered on Jurkat cells by spinoculation, as has been described[38]. Efficiency of infectivity was determined based on %GFP expression using a Gauva EasyCyte flow cytometer. For subsequent experiments, the %-infected was used to calculate the volume of RV SN needed to obtain 99% transduction efficiency for a fixed cell number. For all experiments reported here, the transduction efficiency was greater than 94%. Dual transductions with pHSPG-Siva and pHSPG-FOXP3 RV were performed as follows. A fixed number of cells was transduced with either pHSPG or pHSPG-Siva on day 0. Two days later, high transduction efficiency was confirmed, the cells were counted and equal cell numbers were distributed and mixed with RV for either pHSPG or pHSPG-FOXP3. Since both sets of RV use GFP to mark transduction efficiency, $GFP^{neg}$ Jurkat cells were transduced in parallel to control for transduction efficiency.

*IL-2 ELISA*

To evaluate endogenous IL-2, transduced Jurkat T cells were distributed in 96-well plates at a concentration of $2x10^5$ cells/well. Cells were activated with phorbol 12-myristate

13-acetetate (PMA, 25 ng/ml) and Ionomycin (1 μM). At the end of ~18 hours, cell viability was evaluated on a Gauva EasyCyte. Plates were spun down and supernatants were collected and stored at -20°. IL-2 concentrations were determined with a BD OptEIA™ Mouse IL-2 ELISA kit.

*Jurkat transfections and luciferase reporter assays*

In order to perform luciferase assays, Jurkat cells were transfected by electroporation in cytomix buffer[42]. Jurkat cells were harvested, counted and redistributed to yield $5x10^6$ cells/transfection. Cells were washed once in PBS before being suspended in cytomix buffer supplemented with 2 mM ATP at time of use (300 μl buffer/transfection). While cells were being washed, plasmid DNA was prepared: 1 μg reporter plasmid (see *Plasmids* above) was mixed with differing amounts of plasmid expressing genes of interest or the vector control (10 μg DNA total). Cells in cytomix buffer were mixed with DNA mixtures and transferred to cuvettes on ice (Biorad Gene Pulser Cuvette, 0.4 cm). A Biorad Gene Pulser Xcell was used to electroporate cells at 300V, 500μF, and infinite resistance. Immediately following electroporation of all samples, the shocked cells were diluted in pre-warmed culture media (RPMI 10%FBS described above). Cells were cultured in 6-well plates. After resting for 12-24 hours, cells were distributed into 96-well plates and activated with PMA, 25 ng/ml and ionomycin, 1 μM. Duration of activation ranged from 6-12 hours. At the assay endpoint, 20 μl/well was removed to measure viability and transfection efficieny on Gauva EasyCyte mini flow cytometer. Plates were spun and cells were washed once with PBS to remove growth media. Cells were lysed in 45 μl 1X Promega Reporter Lysis Buffer and frozen at -80°. Plates were read on a Veritas Microplate Luminometer according to the manufacturer's instructions.

*Statistical analysis*

A two-tailed Student's t-test was used to evaluate statistical difference between experimental treatments and a p-value<0.05 was set as the cutoff for statistically significant difference.

## RESULTS

### *Siva physically interacts with FOXP3*

A yeast two-hybrid screen for FOXP3-binding partners was performed with a human thymus cDNA library. Six full-length clones for *Siva-1* were identified in the screen. We chose *Siva-1* for further investigation for two reasons. First, *Siva* regulates T cell apoptosis and, second, because Siva associates with TNFR family members that might contribute to $T_{reg}$ function: CD27, GITR, and OX40[29-33].

After confirming the interaction in yeast, we tested whether FOXP3 and Siva could interact in mammalian cells. Two isoforms, Siva-1 and Siva-2, have been described and their protein domain organization is depicted in Fig 2.1A. Siva-2 lacks the second exon, resulting in a protein that is missing most of the spherical amphipathic helix (SAH) and death domain homology region (DDHR). We performed co-immunoprecipitation (Co-IP) experiments with transfected 293T cells. We transfected 293T cells with expression plasmids for EGFP-tagged Siva isoforms and Myc-tagged FOXP3, or the appropriate vector control. Myc/FOXP3 was immunoprecipitated (IP'ed) with a mouse anti-Myc hybridoma supernatant, 9E10. As shown in Figure 2.1B, by western blotting (WB) with antibody against GFP, we detected co-IP of Siva-1 and Siva-2 in the presence of Myc/FOXP3, but not in its absence. EGFP alone did not co-IP with FOXP3 (Data not shown). Thus both Siva-1 and Siva-2 specifically interact with FOXP3.

### *Siva is preferentially expressed in $CD4^{pos}CD25^{pos}$*

Next, we investigated *Siva* expression in human $CD4^{pos}$ T cells under different stimulation conditions. We measured *Siva* expression by RT-PCR in $CD4^{pos}CD25^{neg}$ and

$CD4^{pos}CD25^{pos}$ T cells. Further, we activated some of the isolated cells for 24-hours. As shown in Figure 2.2A, *Siva* mRNA levels were ~30-fold higher in freshly isolated $CD25^{pos}$ cells compared to $CD25^{neg}$ CD4 T cells. The data supports the possibility that human $T_{regs}$ may be enriched for *Siva* compared to naïve $CD4^{pos}$ T cells. While freshly isolated naïve $CD4^{pos}CD25^{neg}$ cells expressed less *Siva* compared to $CD4^{pos}CD25^{pos}$ T cells, activation with α-CD3 and α-CD28 induced *Siva* mRNA expression.

To compare *Siva* expression in mouse T cell subsets, we took advantage of the public gene atlas microarray data from BioGPS[39,40]. Based on the microarray data for mouse $CD4^{pos}$ T cells and $CD4^{pos}Foxp3^{pos}$, Siva expression is slightly increased in the $Foxp3^{pos}$ population (Figure 2.2B).

*Siva and FOXP3 co-localize in the nucleus*

Observations in $Foxp3^{GFP}$ mice and in transiently transfected cells suggest constitutive nuclear localization of Foxp3[33,43]. In contrast, western blot analysis of nuclear and cytoplasmic extracts indicated a minor Foxp3 presence in the cytoplasm[44]. Siva's localization pattern seems to be dependant on the cell type, the presence or absence of certain binding partners, and the differentiation state of the cell (Summarized in Table 1.2)[45-52].

In order to examine whether Siva and FOXP3 would colocalize to similar cellular compartments, we stained U2OS cells transfected with EGFP/Siva and Myc/FOXP3 (Figure 2.3). The majority of cells coexpressing both proteins showed diffuse staining throughout the nucleus. Also, a small fraction of Siva expressing cells showed a punctate staining pattern that was present in the absence of FOXP3; FOXP3 did not show punctate distribution in the absence of Siva.

*Siva-binding activity is contained within FOXP3's central region*

Major domains of the FOXP3 protein are associated with specific functional activities and binding partners (Figure 2.4)[12]. The Foxp3 N-terminus is required for NFAT-mediated repression of *IL-2*[22] and is involved in binding to the RORγt and RORα transcription factors[19]. The leucine zipper (L.zip) domain mediates homo-dimerization of Foxp3, which contributes to $T_{reg}$ suppressive function[11,21]. The C-terminal Foxp3 forkhead (Fkh) domain is required for DNA binding and nuclear localization[26]. A short stretch of amino acids between the zinc finger (ZnF) and the Fkh domain is involved in AML-1/RUNX1 binding and is involved in *IL-2* repression[15].

We used Myc/FOXP3 truncation mutants to characterize the region involved in binding to Siva by co-IP in 293T cells (summarized in Figure 2.4A). Mutants lacking the Fkh domain (ΔFkh) or the N-terminus (Δ1-71; Δ1-105) bound to Siva with similar efficiency and the Fkh domain alone failed to pull down Siva (Figure 2.4B). Thus, the Siva-binding capacity of FOXP3 is located within the central region that spans the leucine zipper, zinc finger and AML1/RUNX1-binding domains (AAs 106-334).

*The Siva C-terminus is sufficient for binding to FOXP3*

As shown in Figure 2.5A, we designed Siva truncation mutants fused to EGFP's C-terminus to cover major domains that have been previously described and associated with functional effects (summarized in Table 1.3). We performed co-IPs to map Siva's FOXP3-binding domain. Our analysis definitively showed no interaction between FOXP3 and Siva mutants lacking the C-terminus (Figure 2.5 B). In contrast, all mutants containing some portion of the cysteine rich Siva C-terminal domain interacted with FOXP3. Given that the

Siva C-terminus mutant and the ΔZnF mutant both contain the B box domain and both interacted with FOXP3, we hypothesized that the Siva B box domain might be necessary and sufficient to bind FOXP3. To test our hypothesis, we sub-cloned new mutants encompassing the Siva B box domain and full length Siva lacking the B box domain (ΔB box). Subsequent co-IP experiments demonstrated that the Siva B box domain is neither necessary nor sufficient to bind FOXP3 (Figure 2.5C).

*Siva negatively regulates IL-2 gene expression*

We next investigated Siva function. In order to evaluate the effect of *Siva* overexpression on endogenous IL-2, we transduced Jurkat T cells with pHSPG-Siva-1 RV. Two pHSPG-Siva-1 plasmid clones were evaluated for Siva-1 protein expression, transduction efficiency and endogenous IL-2 expression in Jurkat cells (Figure 2.6 *A-C*). The rabbit α-Siva FL-175 antibody detected a prominent band from 293T cell lysates transfected with pEGFP/Siva, but not in the control lanes. Overexpression of Siva-1 by retroviral transduction repressed endogenous IL-2 by ~40-60% compared to pHSPG vector-transduced cells.

Next, we tested the effect of *Siva* knockdown (KD) on endogenous IL-2 gene expression (Figure 2.6D). Jurkat T cells were transduced with pLKO lentivirus (LV) expressing *shSiva* or *shEGFP*, as a control shRNA. *Siva* KD enhanced IL-2 expression by five fold compared to the *shEGFP* control.

*Siva negatively regulates IL-2 promoter activity*

In addition to measuring *Siva's* effect on IL-2 protein levels, we tested the effect of *Siva* on *IL-2* promoter activity. A minimal enhancer element located 300 base pairs

immediately upstream of the *IL-2* start site regulates transcription through the binding of transcription factors and alterations in the chromatin structure[53,54]. Distal regulatory elements also contribute to *IL-2* regulation and the minimal enhancer region is sometimes referred to as the proximal promoter. In order to investigate *Siva's* effect on *IL-2* transcriptional activation, we used a reporter plasmid containing the *IL-2* proximal promoter driving expression of the luciferase enzyme. Overexpression of *Siva* inhibits *IL-2* transcriptional activity (Figure 2.7 A) and KD of *Siva* enhances *IL-2* transcription in a dose-dependent manner (Figure 2.7 B).

*FOXP3 and Siva repress endogenous IL-2*

In order to test whether *FOXP3* and *Siva* functionally interact to repress endogenous IL-2, we expressed *FOXP3* and *Siva* in Jurkat cells by transduction with pHSPG RVs (Figure 2.8A). As expected, *FOXP3* and *Siva* independently repressed endogenous IL-2 (Figure 2.8B). The presence of *Siva* appeared to inhibit the repressive ability of *FOXP3,* but the difference was not statistically significant (Figure 2.8C). We also examined the combined effects of *Siva* KD and exogenous *FOXP3* on IL-2 (Figure 2.9). KD of endogenous *Siva* did not significantly enhance the repressive effect of *FOXP3* (Figure 2.9C). However, the presence of *FOXP3* slightly, but significantly impaired the IL-2-enhancing effect of *Siva* KD (Figure 2.9D).

In summary, we observed a trend whereby *FOXP3's* ability to repress IL-2 gene expression is inversely correlated with the presence of *Siva.* Similarly, in Siva-KD cells, *FOXP3* showed greater activity in suppressing the IL-2 gene.

*Effect of FOXP3 and Siva overexpression on IL-2 promoter activity*

After exploring the hypothesis that *FOXP3* and *Siva* functionally interact to repress endogenous IL-2, we hypothesized that *FOXP3* and *Siva* might functionally interact to repress *IL-2* transcriptional activity. First, we tested the effect of *Siva* and *FOXP3* overexpression on transcription of the *IL-2* proximal promoter in a Jurkat T cell luciferase assay. We transfected Jurkat T cells with the *IL-2* reporter plasmid and different combinations of pHSPG vector, a fixed amount of pHSPG-Siva and a titrated amount of pHSPG-FOXP3. As indicated by the data in Figure 2.10A, both *Siva* and *FOXP3* repress *IL-2* transcriptional activity. The data shown in Figure 2.10A was used to calculate FOXP3 mediated IL-2 repression in the presence and absence of Siva. In Figure 2.10B, the "vector" (dark diamonds) data points were calculated by setting the IL-2 RLU for PG/vector (Figure 2.10A, first dark bar) as 100% *IL-2* luciferase activity; the "Siva" (light squares) data points were calculated by setting the IL-2 RLU for PG/Siva (Figure 2.10A, first grey bar) as 100% *IL-2* luciferase activity. FOXP3's *IL-2* repressive effect is illustrated by the decline in %IL-2 luciferase activity with increasing doses of *FOXP3.* At lower doses of FOXP3, Siva's presence inhibits FOXP3's repressive effect on IL-2 luciferase activity (Figure 2.10B, square compared to diamond). However, when both genes are overexpressed at a one-to-one ratio, *Siva* does not alter *FOXP3* repression of *IL-2* transcription.

*Effect of FOXP3 overexpression and Siva knockdown on IL-2 promoter activity*

As shown above in Figure 2.6D, Jurkat cells express endogenous *Siva* and endogenous *Siva* KD enhances *IL-2 promoter* activity. We hypothesized that endogenous *Siva* might affect *FOXP3's IL-2* repressive ability. We tested the effect of combined *Siva* KD

and *FOXP3* overexpression on *IL-2* promoter-driven luciferase activity (Figure 2.11A). *Siva* KD enhanced the repressive effect of *FOXP3* on *IL-2* transcriptional activity (Figure 2.11B). *FOXP3* impaired the *IL-2*enhancing effect of *Siva* KD (Figure 2.11C).

*Effects of FOXP3 and Siva on NFκB, NFAT, and AP-1*

*IL-2* is regulated by multiple transcription factors and chromatin modifications[53,54]. We tested the effects of *Siva* and *FOXP3* on three major *IL-2* transcriptional regulators. Consistent with published reports and shown in Figures 2.12 A-C, *FOXP3* inhibits NFκB[13], NFAT[13,22], and AP-1[16] transactivation potential in stimulated Jurkat T cells. *Siva* repressed NFκB, but had no effect on NFAT or AP-1 activity in response to stimulation. Interestingly, *Siva* repressed basal level AP-1 activity in the absence of stimulation (Figure 2.12D). The repressive effects of *FOXP3* and *Siva* on NFκB activity parallel the effects of both genes on *IL-2*.

*Effects of FOXP3 and Siva on Jurkat T cell viability*

Next, we hypothesized that *FOXP3* might alter the well characterized pro-apoptotic effect of *Siva.* We measured cell viability by 7AAD in Jurkat T cells transduced with both pHSPG-Siva and pHSPG-FOXP3 RV (transduction efficiency shown in Figure 2.8A). As expected, *Siva* enhanced Jurkat T cell death in the absence of stimulation (Figure 2.13). In all experiments we observed that *FOXP3* also marginally enhanced apoptosis in unstimulated cells, but this effect was not always statistically significant. *FOXP3* did not alter the death promoting effect of *Siva* in the absence of stimulation.

Jurkat T cell apoptosis is induced by exposure to PMA and ionomycin for ~18 hours. Under these PMA/ionomycin treatment conditions, the pro-apoptotic effect of *Siva*

overexpression was small, but statistically significant (Figure 2.14). Interestingly, *FOXP3* protected Jurkats from PMA/ionomycin-induced apoptosis. Fold-change analysis of cell death measurements did not indicate an antagonistic effect between *Siva* and *FOXP3* (data not shown). Finally, the stimulation-dependent effect of *FOXP3* on cell viability is highlighted in Figure 2.15. *FOXP3* enhanced apoptosis in the absence of stimulation, but protected Jurkat T cells from apoptosis in the presence of PMA and ionomycin.

## DISCUSSION

In this report we have shown that *FOXP3* and *Siva* physically interact. We have mapped the binding activities to limited domains within each protein. Both *FOXP3* and *Siva* repressed *IL-2* gene expression. One likely mechanism by which *Siva* represses *IL-2* is via inhibition of NFκB. *FOXP3* repressed the activity of three *IL-2* transactivators: NFκB, NFAT, and AP-1. *FOXP3* did not affect Siva's pro-apoptotis effect. An interesting effect was observed whereby *FOXP3* enhanced apoptosis in the absence of stimulation, but protected from apoptosis induced by PMA and ionomycin.

The physical interaction was demonstrated through a standard protein-protein interaction, co-immunoprecipitation assay. Two Siva isoforms have been identified and both Siva isoforms interact with FOXP3. The Siva-2 isoform lacks the second exon that encodes for the SAH and DDHR domains[28,45]. Both Siva isoforms share the C-terminal domain that contains FOXP3-binding capacity. The Siva C-terminus spans the putative B-box and ZnF domains. Siva retains FOXP3-binding ability when either the Siva B box domain or the ZnF domains are removed.

Efforts were made to detect a biophysical interaction between endogenously expressed FOXP3 and Siva, but these were met with technical challenges. However, failure to detect an interaction does not mean that the interaction does not occur. Also, a functional interaction between *FOXP3* and *Siva* could be independent of the physical, biochemical interaction between the two proteins. In order to test whether the biochemical interaction between *FOXP3* and *Siva* affects their functional interaction, a minimal region of biochemical interaction for at least one protein needs to be identified.

The Siva C-terminus is enriched with cysteine residues that could be important to the tertiary protein structure and function (Figure 2.16). Paired cysteine residues are associated with intramolecular disulfide bond formation. The Siva B-box domain contains four paired cysteine residues. The ZnF domain contains two paired cysteine residues and one, single cysteine residue. Nestler *et al*[55] used truncation mutants to show that Siva coordinates three zinc ions, two of which are associated with the C-terminus[55]. Although a few groups have found that the Siva C-terminus is necessary to interact with other binding partners[48,56,57], no one has described whether specific C-terminal point mutations block Siva function. Site-directed mutagenesis of the Siva C-terminal cysteine residues could be tested for physical interaction with FOXP3 and could be useful towards understanding a possible functional interaction between FOXP3 and Siva.

FOXP3's capacity to bind Siva is contained within a broad central region that includes the ZnF, the L.Zip and the AML1/RUNX1 binding domain (amino acids 106-332). Mutants missing either the first 105 N-terminal amino acids or the C-terminal Fkh domain sustained binding to Siva. Further experiments are needed to define a minimal region of FOXP3 involved in binding to Siva. The Foxp3 L.Zip domain is required for homodimerization, *IL-2* repression, and $T_{reg}$ suppressive function[11,21,43]. Given that the L.Zip domain is within the region that binds Siva, one obvious next step we plan is to test whether known L.Zip IPEX mutations (Δ250, Δ251)[11,21] interfere with FOXP3 binding to Siva.

Even though both *FOXP3* and *Siva* independently repress *IL-2* transcriptional activity, modulation of the expression level of *Siva* affects the repressive effect of FOXP3 on *IL-2*. *Siva* overexpression reduced the relative inhibitory activity of *FOXP3*. Knocking down endogenous *Siva* enhanced *FOXP3's* repressive effect on *IL-2* transcriptional activity. These

data could mean that *FOXP3* and *Siva* functionally interact to repress *IL-2* by affecting similar gene regulatory mechanisms. Such a proposed functional interaction would not necessarily be dependent upon the observed biophysical interaction that we have characterized.

Our analysis of three *IL-2* transactivators supports the assertion that *Siva* and *FOXP3* affect shared and different *IL-2* regulatory pathways. Consistent with previous reports, *FOXP3* repressed NFκB[13,16,22], NFAT[21,36], and AP-1[15] transactivation reporters. *Siva* inhibited NFκB, but had no effect on either NFAT or AP-1 in cells stimulated with PMA and Ion. Our data indicating that *Siva* has no effect on AP-1 in activated Jurkat T cells conflicts with data showing that *Siva* enhanced AP-1 activity[58].

The effects of *FOXP3* and *Siva* on *IL-2* resembles *FOXP3*- and *Siva*-mediated repressive effects observed for the MHC3κB reporter. A small, but significant additive effect was observed when *Siva* and *FOXP3* were added in combination.

The small additive NFκB-repressive effect that we observed between *FOXP3* and *Siva* could be related to cytoplasmic and nuclear NFκB signaling events. In resting cells, cytoplasmic IκBα sequesters the canonical NFκB subunits p65 and p50, preventing their nuclear translocation. In response to stimulus, activated IKK phosphorylates IκBα, triggering ubiquitin-mediated degradation of IκBα and release of the NFκB subunits[59]. One group showed that *FOXP3* binds to the NFκB p65 subunit and inhibits NFκB transactivation potential by mechanisms independent of nuclear translocation and DNA binding[13]. Previously, we observed that *FOXP3* affects NFκB transactivating potential independent of nuclear translocation, but dependent on the promoter sequence. FOXP3 enhanced HIV-1

gene expression by increasing the amount of NFκB p65 subunit bound to the LTR (long terminal repeat)[10]. Thus, the known effect of FOXP3 on NFκB activity occurs downstream of IκBα degradation, whereas Siva is known to inhibit NFκB activity by stabilizing IκBα and by blocking NFκB nuclear accumulation[36]..

Siva may have a transcriptional regulation function within the nucleus as others and we have observed the subcellular localization of Siva to be both nuclear and cytoplasmic[46,48-50,52]. However, as of yet, neither DNA-binding by EMSA nor chromatin binding by chromatin IP assay has been reported for Siva. Even though Siva is present in the nucleus, most detailed reports regarding intracellular Siva function have investigated Siva function in relation to transmembrane cytoplasmic tails or mitochondrial-associated apoptosis regulators present in the cytoplasm, not the nucleus[28,29,45,48,50,56,60-62].

We have shown that freshly isolated $CD4^{pos}CD25^{pos}$ cells expressed *Siva* mRNA and that $CD4^{pos}CD25^{neg}$ cells upregulated *Siva* mRNA in response to TCR stimulation. FOXP3 expression is also upregulated in human naïve CD4 T cells in response to activation[63]. Thus, *Siva* and *FOXP3's* repressive effects on *IL-2* could contribute to either conventional $CD4^{pos}$ T cell function or thymic derived $nT_{reg}$ function.

In the future, we would like to extend our investigations of *FOXP3*- and *Siva*-mediated *IL-2* repression to primary T cells. We plan to evaluate *Siva's* effect on *IL-2* in $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ T cells. Also, another remaining question is whether *Siva* could mediate $T_{reg}$ suppressive function.

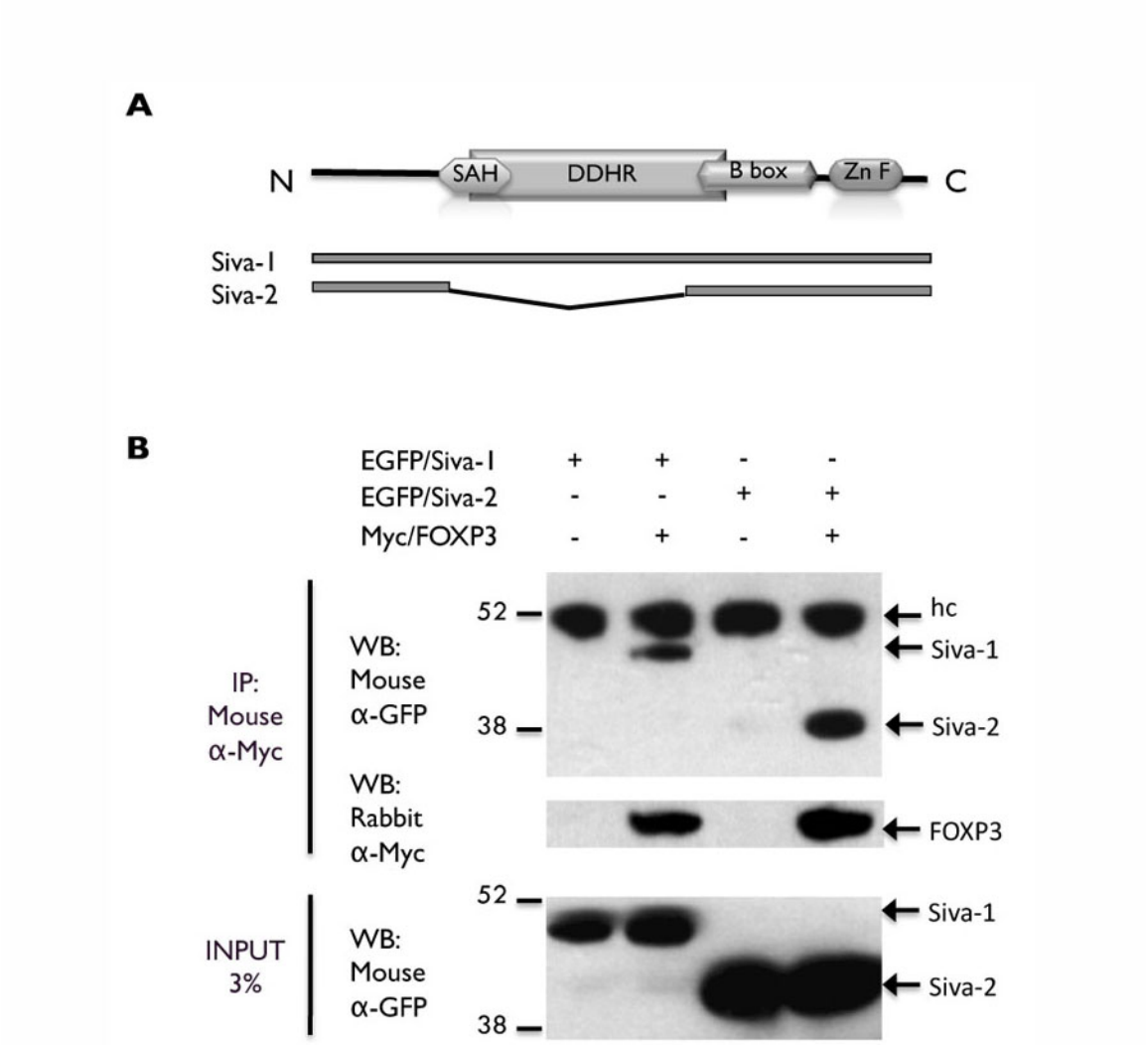


**Figure 2.1: FOXP3 physically interacts with both Siva isoforms.**

A. Diagram showing the Siva protein domain organization [SAH, spherical amphipathic helix; DDHR, death domain homology region; B Box; and ZnF, zinc finger] and a cartoon representation of Siva-1 and Siva-2, which were fused to the C-terminus of EGFP and used in the Co-IP shown in B.

B. FOXP3 interacts with Siva-1 and Siva-2 in 293T cells. Plasmids expressing EGFP/Siva fusion constructs in the presence or absence of Myc/FOXP3 were transfected into 293T cells. Cell lysates were prepared in modified RIPA buffer and protein concentrations were measured to standardize immunoprecipition (IP) reactions (500 μg) and inputs (15 μg). Myc/FOXP3 was IP'ed with a mouse anti-Myc 9e10 hybridoma supernatant. To detect the proteins in IPs and inputs, western blot (WB) was performed with a mouse monoclonal antibody against GFP (Contech, JL-8). Membranes were stripped and reprobed with a rabbit anti-Myc polyclonal antibody. For further details see Methods and Materials, *293T Transfections, Co-IPs, and Antibodies*. Siva-1 binding to FOXP3 was observed in 5 or more experiments. Siva-2 binding to FOXP3 was observed in 3/3 Co-IPs.

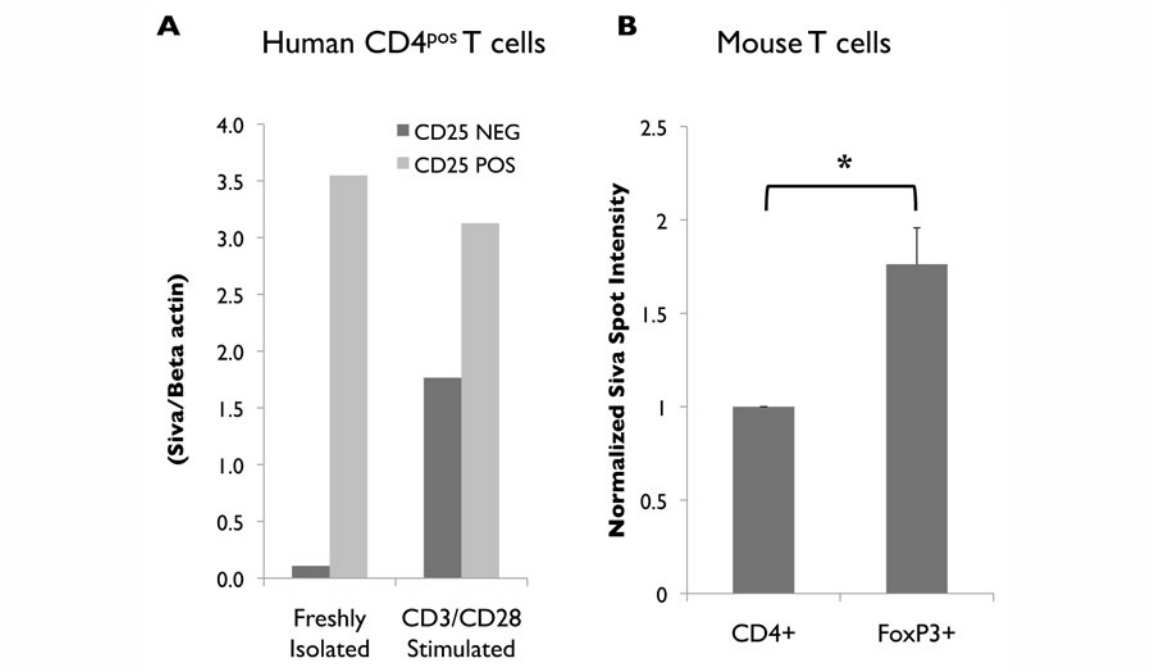


**Figure 2.2: *Siva* expression in human and mouse CD4 T cells.**

A. Expression of *Siva* mRNA in human $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ cells freshly isolated from peripheral blood mononuclear cells or stimulated with α-CD3 and α-CD28 for 24-hours. *Siva* and *β-actin* expression was measured by semi-quantitative RT-PCR. Image quant was used to quantify band intensities and the ratio of *Siva* normalized to *β-actin* is shown. Experiment was performed one time.

B. Normalized *Siva* spot intensity in mouse $CD4^{pos}$ T cells. Data was obtained from the BioGPS Mouse Gene Atlas. Spots were probed with RNA from $CD4^{pos}$ and $Foxp3^{pos}$ T cells. The spot intensity raw values from two *Siva* spots on the array were normalized to the respective spot value for $CD4^{pos}$ T cells. For more information see Methods and Materials, *BioGPS Data Analysis.* The normalized data was evaluated with a two-tailed Student's t-test and * indicates the p-value < 0.05.

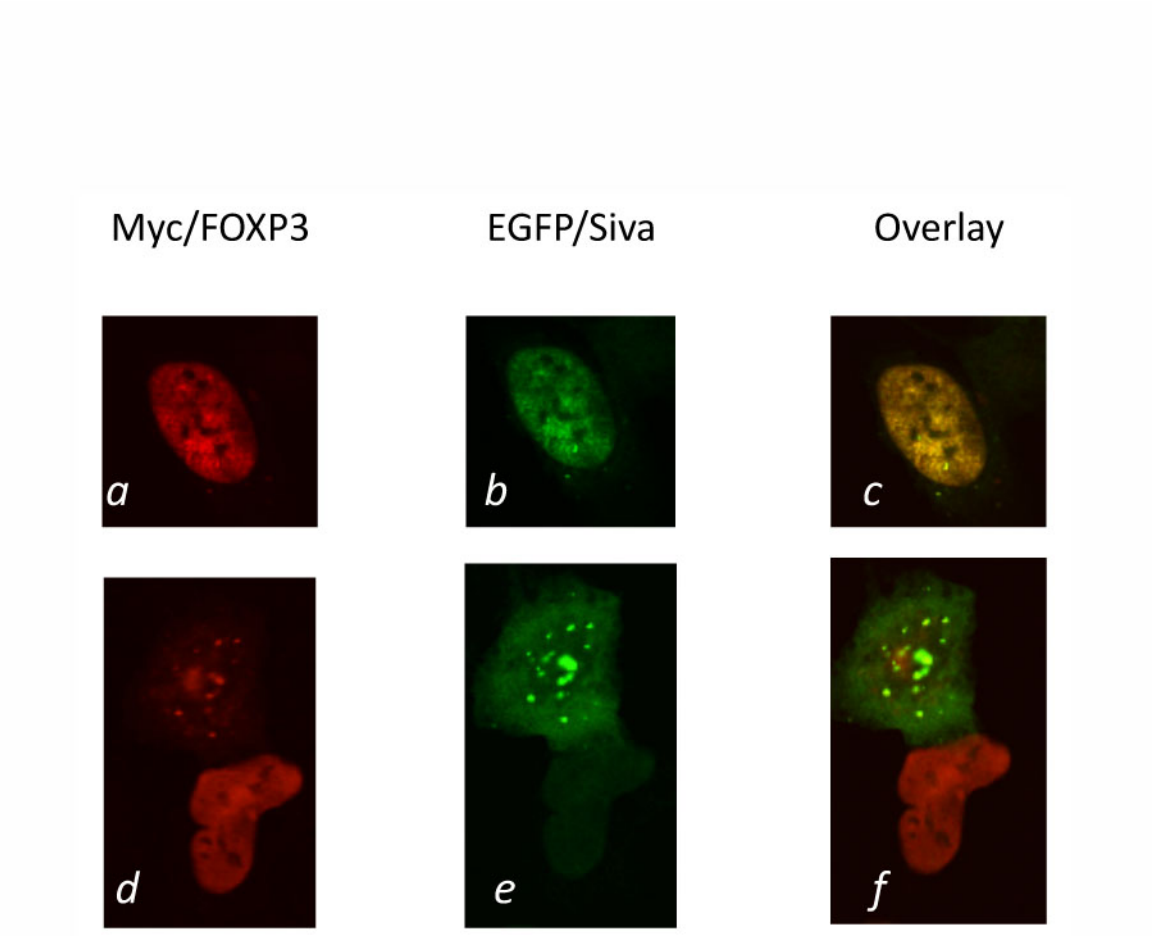


**Figure 2.3: Siva-1 and FOXP3 co-localize in the nucleus of U2OS cells.**
U2OS cells were plated in chamber slides and co-transfected with plasmids expressing Myc/FOXP3, EGFP/Siva, and/or vector controls. Negative transfection controls and staining controls are not shown. Transfected cells were stained with Rhodamine-conjugated anti-Myc or Rhodamine-conjugated IgG isotype as a negative control. The same cell is shown in *a-c*; two adjacent cells are shown in *d-f.* Rhodamine α-Myc detection of Myc/FOXP3 (*a,d);* EGFP/Siva (*b,e);* Overlay (*c,f).* Printed with permission from Dr. Stephanie Mackey Cushman.

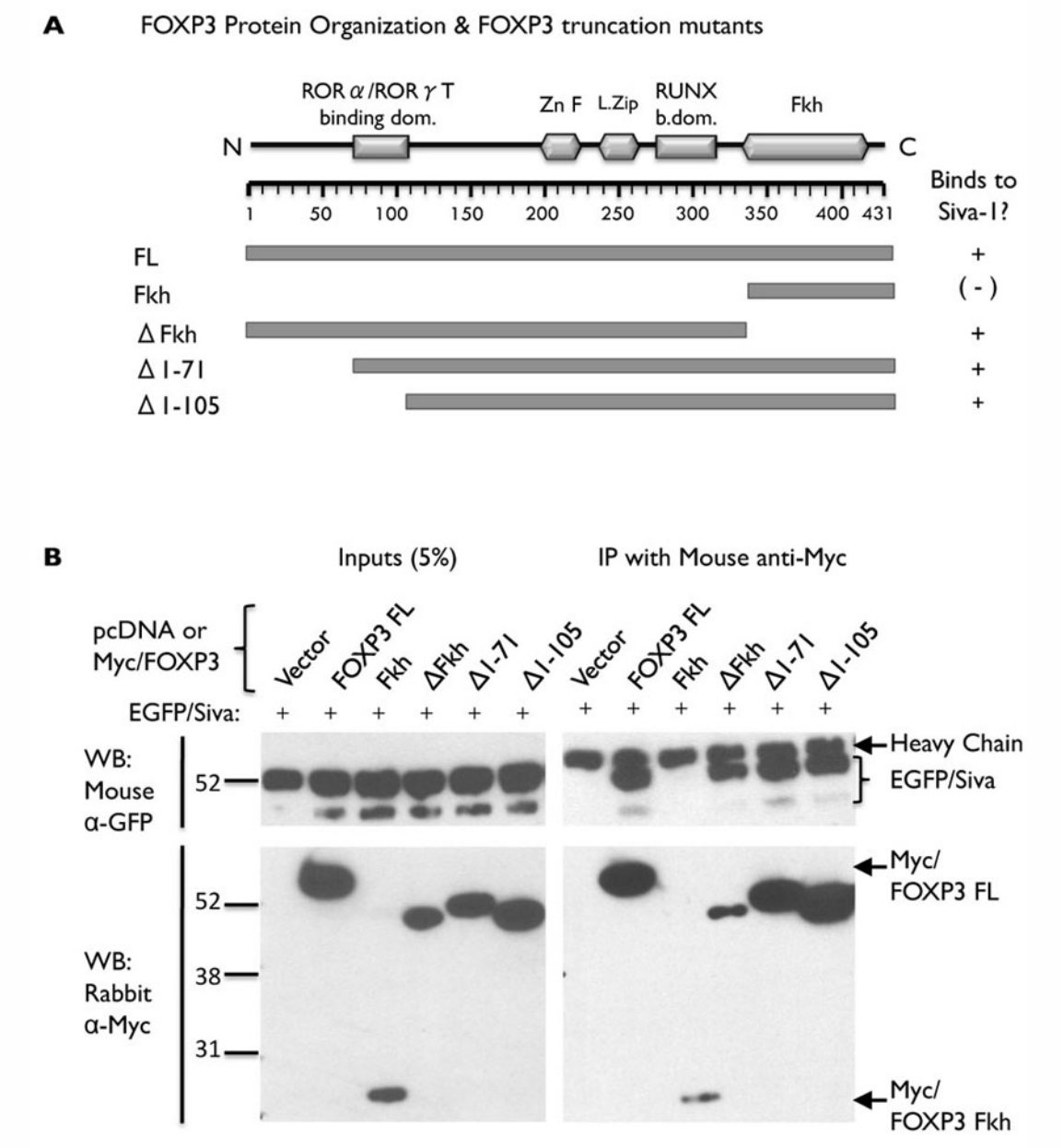


**Figure 2.4: Siva-binding activity is contained within a central portion of the FOXP3 protein (amino acids 106-332)**

A. Diagram depicting FOXP3 protein domain organization and truncation mutants that were fused to the C-terminus of Myc and used in the Co-IP shown in B. (Zn F, zinc finger; L.Zip, leucine zipper; b.dom, binding domain; Fkh, forkhead)

B. EGFP/Siva-1 co-immunoprecipitates (Co-IPs) with full-length FOXP3 and FOXP3 truncation mutants containing the ZnF, L.Zip, and AML-1/RUNX1-binding domains. The FOXP3 Fkh domain and N-terminus are dispensable for binding to Siva. Co-IPs were performed as described in Figure 2.1. (WB, western blot). Binding between the Fkh FOXP3 mutant and Siva was tested three times. Other FOXP3 mutants shown here were tested once.

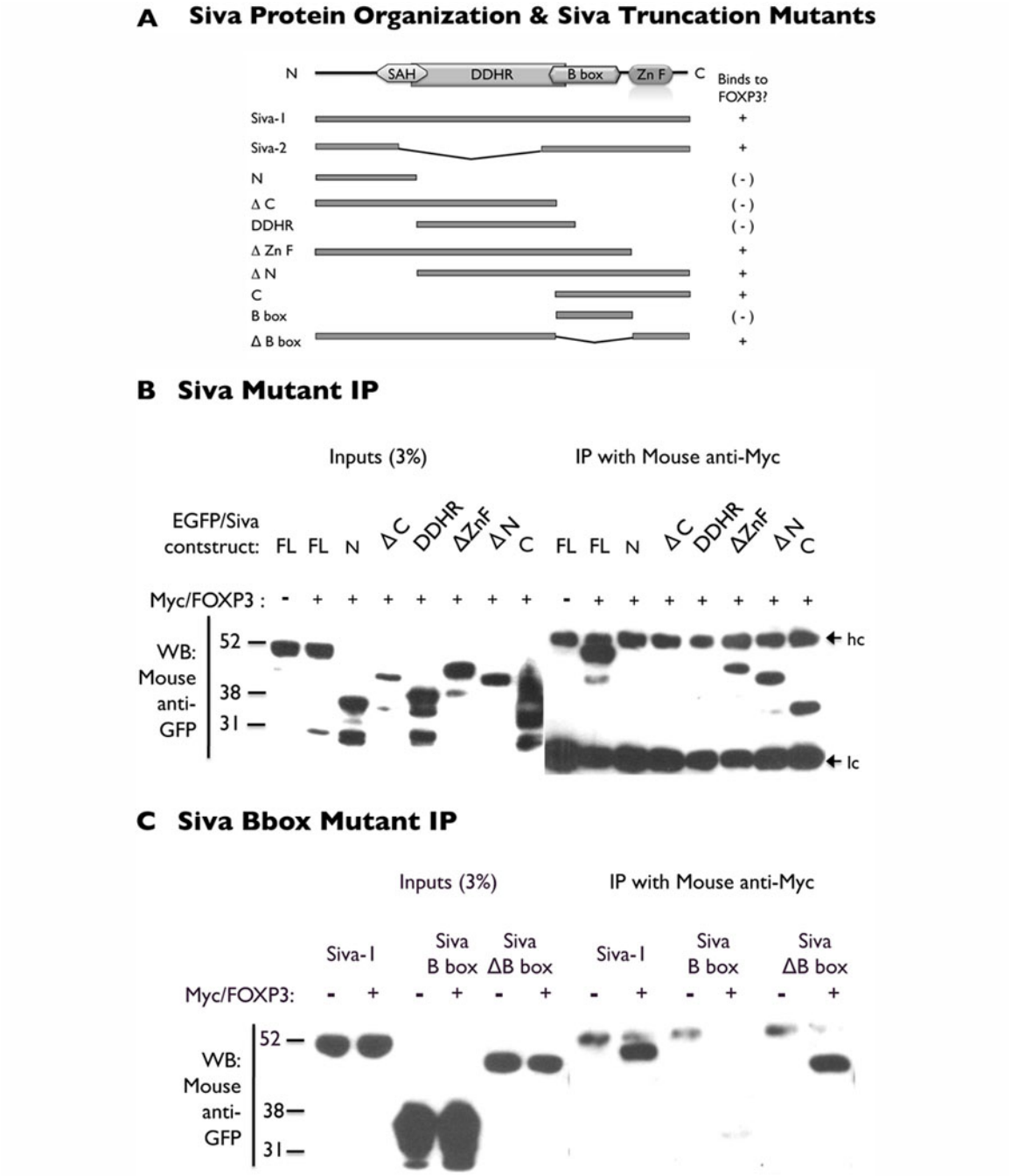


**Figure 2.5: The Siva C-terminus is sufficient to bind FOXP3.**
For experiments shown in B and C, see the Co-IP procedure described in Figure 2.1.

A. Diagram showing the Siva protein organization and Siva truncation mutants, which were fused to the EGFP C-terminus and tested in Co-IPs shown in B and C.
B. Siva mutants containing portions of the C-terminus Co-IP with Myc/FOXP3. These Siva truncation mutants were tested 3 times for FOXP3-binding activity.
C. Co-IP to test the FOXP3-binding capacity of the Siva B-box and ΔB-box mutants. These mutants were tested twice for FOXP3-binding activity.

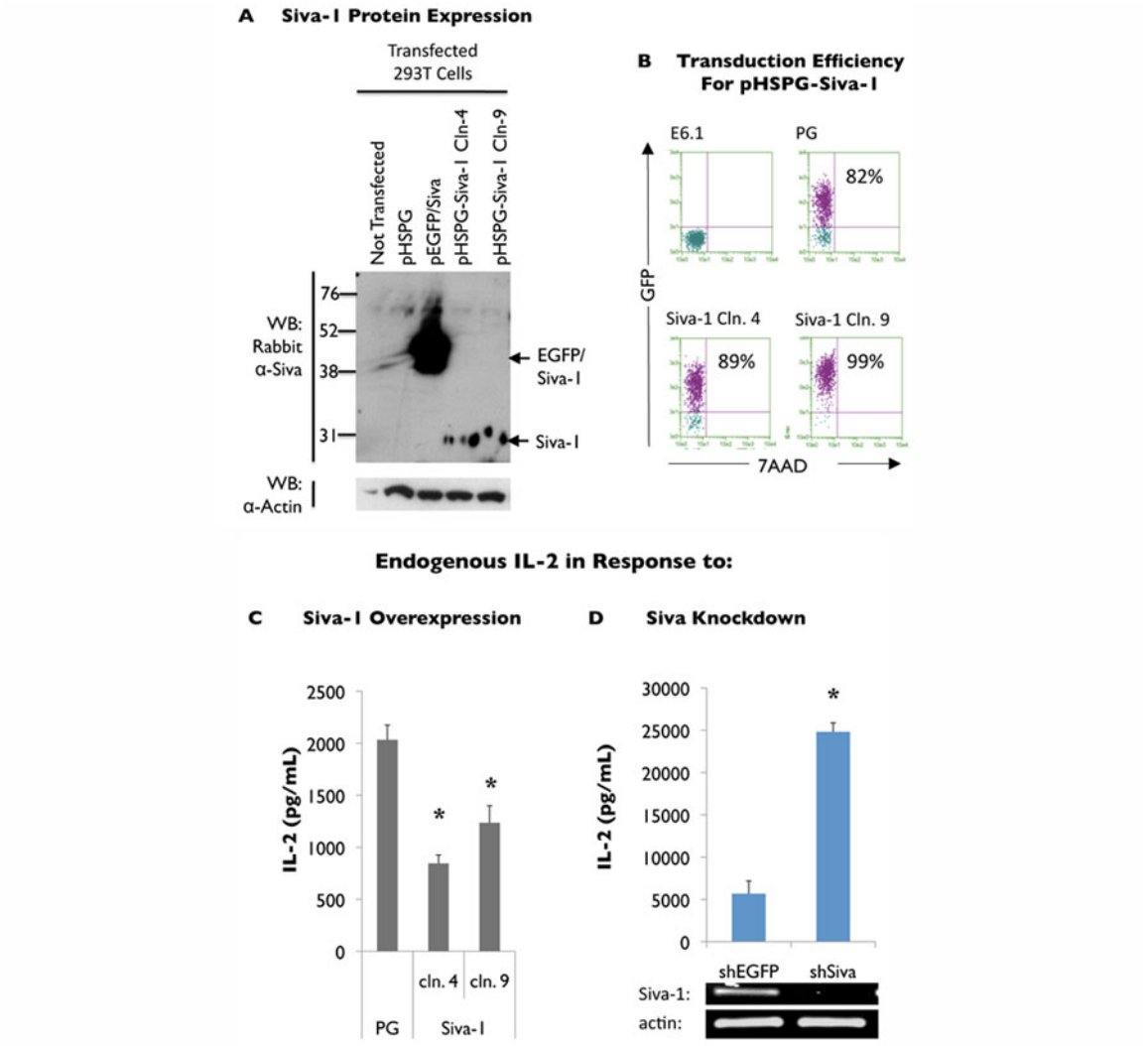


**Figure 2.6: Siva represses endogenous IL-2.**
*Siva* was subcloned into the pHSPG (PG) retroviral (RV) vector. pHSPG-Siva RV was prepared and used to transduce Jurkat T cells that were subsequently used for experiment shown in C. For C and D, transduced, Jurkat T cells were activated with PMA and Ion for 18 hours. Endogenous IL-2 expression was measure by ELISA.

A. Characterization of pHSPG-Siva by western blot (WB) of transfected 293T cell lysates with rabbit anti-Siva (FL-175, Santa Cruz).
B. Evaluation of pHSPG transduction efficiency by flow cytometry for GFP expression in Jurkat T cells. Plots shown were gated on $FSC^{hi}7AAD^{neg}$ cells.
C. Endogenous IL-2 expression for the pHSPG-Siva transduced Jurkat T cells shown in *B*. * indicates $p<0.05$ for two-tailed Student's t-test for *Siva*-transduced cells compared to (PG) controls. Experiment was performed once.
D. Endogenous IL-2 expression for Jurkat T cells transduced with pLKO lentivirus (LV) containing U6-driven shRNA for EGFP or Siva. Cells were selected in puromycin (μg/ml) for one week. Standard RT-PCR evaluated Siva knockdown efficiency and DNA bands are shown. * indicates $p<0.05$ for two-tailed Student's t-test between shEGFP and shSiva. Experiment was performed >5 times.

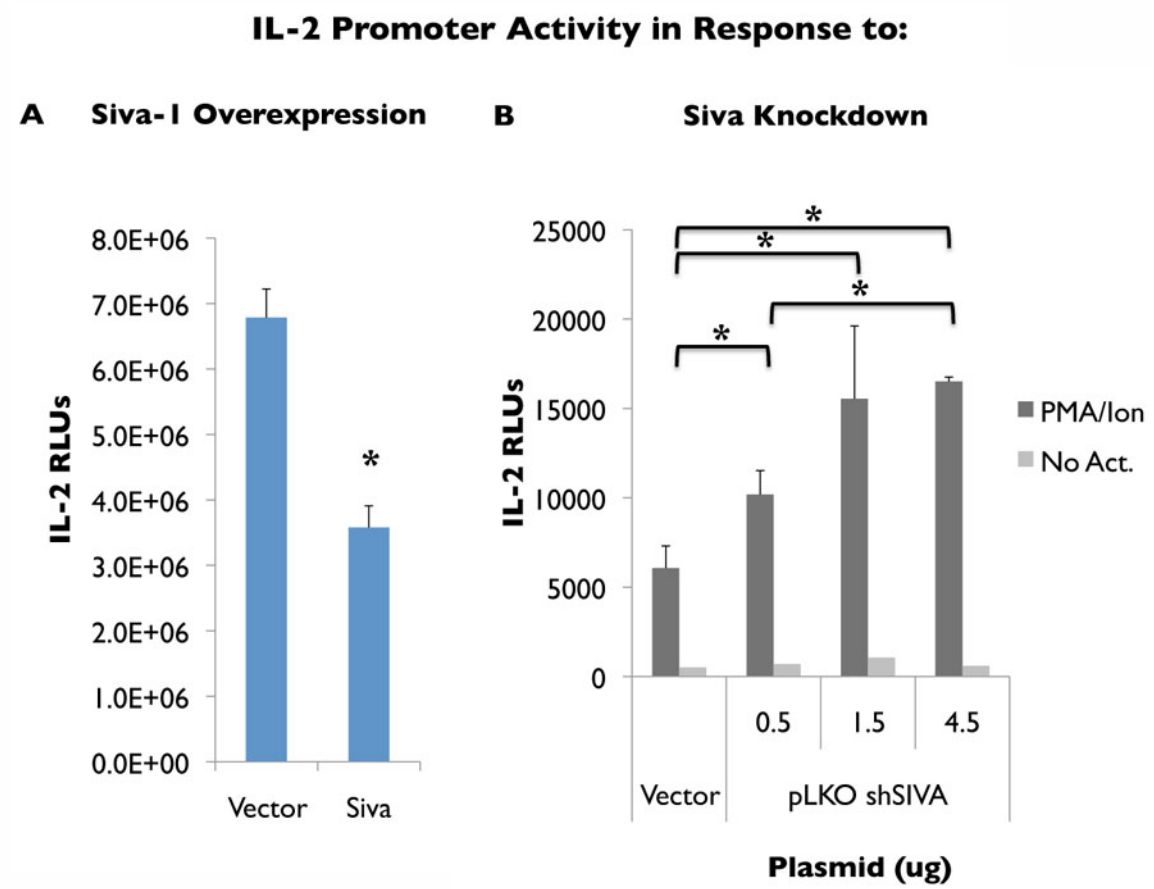


**Figure 2.7: Siva represses *IL-2* promoter activity.**
To evaluate *IL-2* promoter activity, Jurkat T cells were transfected by electroporation with an *IL-2* reporter plasmid (*IL-2* proximal promoter upstream of luciferase) plus other constructs to overexpress or knockdown (KD) *Siva* expression. After recovering from the electroporation for ~18 hours, transfected Jurkat T cells were activated with PMA and Ion for 6 hours. *IL-2* driven luciferase activity in the cell lysates was measured on a luminometer. For more details, see Methods and Materials, *Jurkat Transfections and Luciferase Reporter Assays*.

A. Siva overexpression with pHSPG-Siva repressed *IL-2*-promoter activity. * indicates $p<0.05$ for two-tailed Student's t-test between vector and *Siva* transfected cells.
B. Siva KD by shRNA had an enhancing, dose-responsive effect on *IL-2* promoter activity. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test.

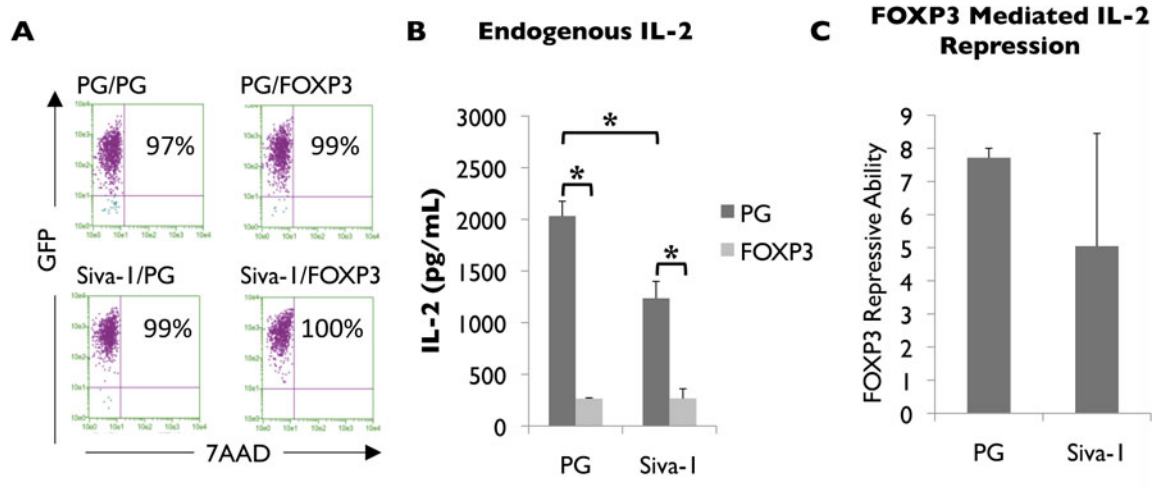


**Figure 2.8: Endogenous IL-2 in response to *FOXP3* and *Siva* overexpression.**
Jurkat T cells were transduced twice: first with pHSPG or pHSPG-Siva RV, then with pHSPG or pHSPG-FOXP3. (See Methods and Materials, *Retrovirus Production and Transduction*). All data shown was derived from the same experiment.

A. Flow cytometry for GFP expression in Jurkat cells transduced with pHSPG-derived RV expressing *FOXP3* and/or *Siva.* Plots shown were gated on $FSC^{hi}7AAD^{neg}$ cells.
B. Endogenous IL-2 in response to *FOXP3* and *Siva* overexpresion. Dual transduced, Jurkat T cells were activated with PMA and Ion for 18 hours. Endogenous IL-2 expression was measure by ELISA. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test.
C. FOXP3-mediated IL-2 repression was determined by calculating fold repression in the presence and absence of *Siva.* Ex. (PG:PG)/(PG:FOXP3) vs. (Siva:PG)/(Siva:FOXP3), using the IL-2 concentrations shown in B.

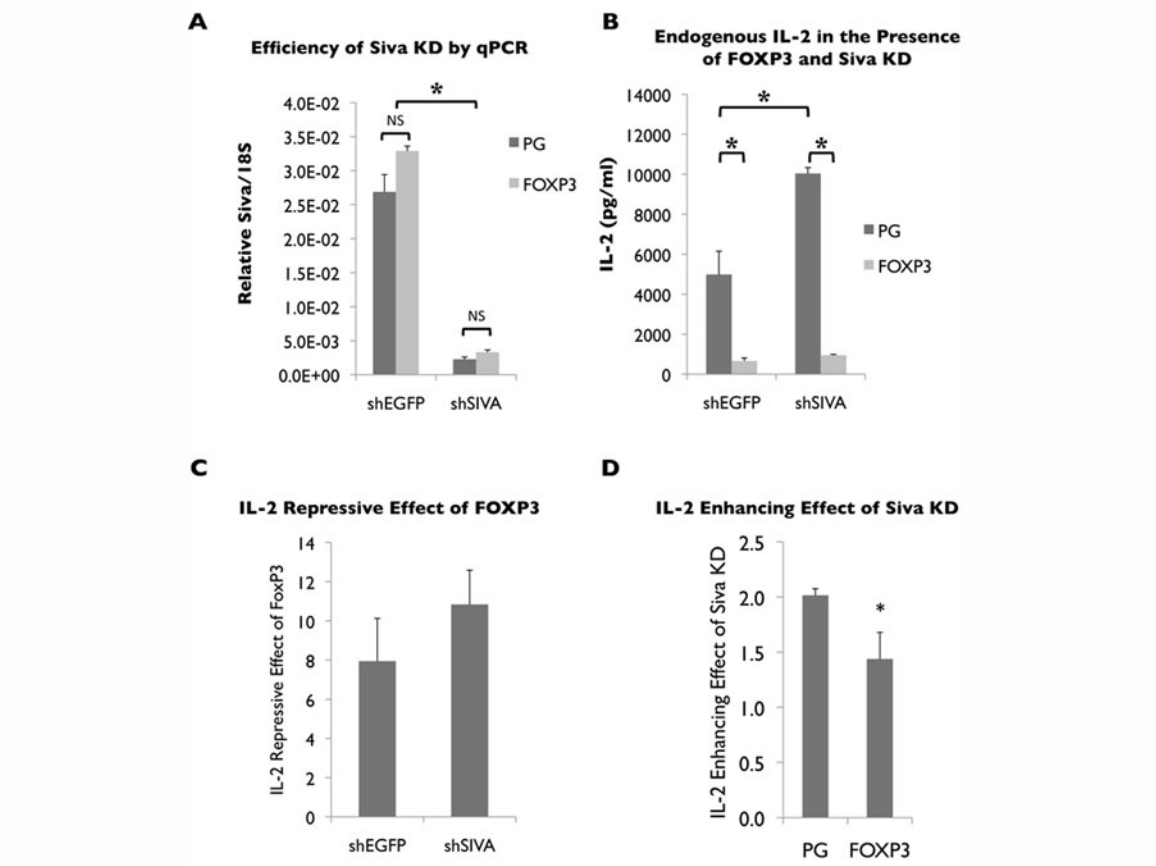


**Figure 2.9: Endogenous IL-2 in response to *FOXP3* overexpression and *Siva* Knockdown (KD).**

First Jurkat T cells were transduced with pLKO-shEGFP or pLKO-shSIVA LV and then selected in puromycin for ~1 week. Next, the same cells were transduced with pHSPG or pHSPG-FOXP3 RV. (See Methods and Materials, *Retrovirus Production and Transduction*). All data shown was derived from the same experiment, which was performed 3 times. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test.

A. *Siva* KD efficiency was determined by quantitative realtime PCR for *Siva* and 18S. cDNA was prepared from mRNA isolated from each cell line. The cDNA template was diluted 1:1000 for 18S reactions. Relative quantities were determined by correlating $C_T$ values to a standard curve. Data shown is the relative quantity of *Siva* normalized to relative quantity of 18S.
B. Endogenous IL-2 in response to *FOXP3* and *Siva* KD. Dual transduced Jurkat T cells were activated with PMA and Ion for 18 hours. Endogenous IL-2 expression was measure by ELISA.
C. The IL-2-repressive effect of *FOXP3* was determined by calculating IL-2 fold repression in the presence or absence of *Siva* KD. Example calculation: (shEGFP:PG)/(shEGFP:FOXP3) vs. (shSiva:PG)/(shSiva:FOXP3) using IL-2 concentrations from B.
D. The IL-2-enhancing effect of *Siva* KD was determined by calculating IL-2 fold enhancement in the presence and absence of *FOXP3* overexpression. Example calculation: (shSiva:PG)/(shEGFP:PG) vs. (shSiva:FOXP3)/(shEGFP:FOXP3).

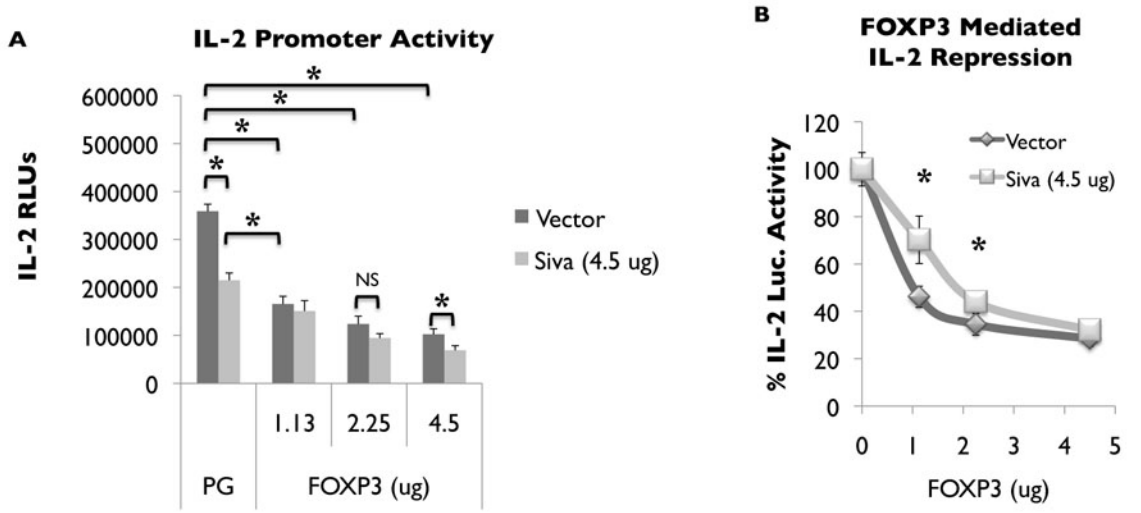


**Figure 2.10: *IL-2* promoter activity in response to *FOXP3* and *Siva* overexpression.** *IL-2* promoter activity was evaluated using a luciferase reporter driven by the *IL-2* proximal promoter, similar to the methods described in Figure 2.7. In addition to the *IL-2 luciferase* reporter plasmid, Jurkat T cells were transfected with Vector (pHSPG) or pHSPG-Siva and titrated amounts of pHSPG-FOXP3. Experiment was performed once.

A. *IL-2* promoter activity in response to *FOXP3* and *Siva* overexpression. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test.
B. *FOXP3*-mediated *IL-2* repression is inhibited by the presence of *Siva* when the *FOXP3* dose is not maximized. "Vector" (dark diamonds) data points were calculated by setting the IL-2 RLU for PG/vector (first dark bar from A) as 100% *IL-2* luciferase activity. "Siva" (light squares) data points were calculated by setting the IL-2 RLU for PG/Siva as 100% *IL-2* luciferase activity. * indicates $p<0.05$ for Student's t-test comparing *FOXP3's* repressive effect in the presence of vector or *Siva* (diamonds vs. squares).

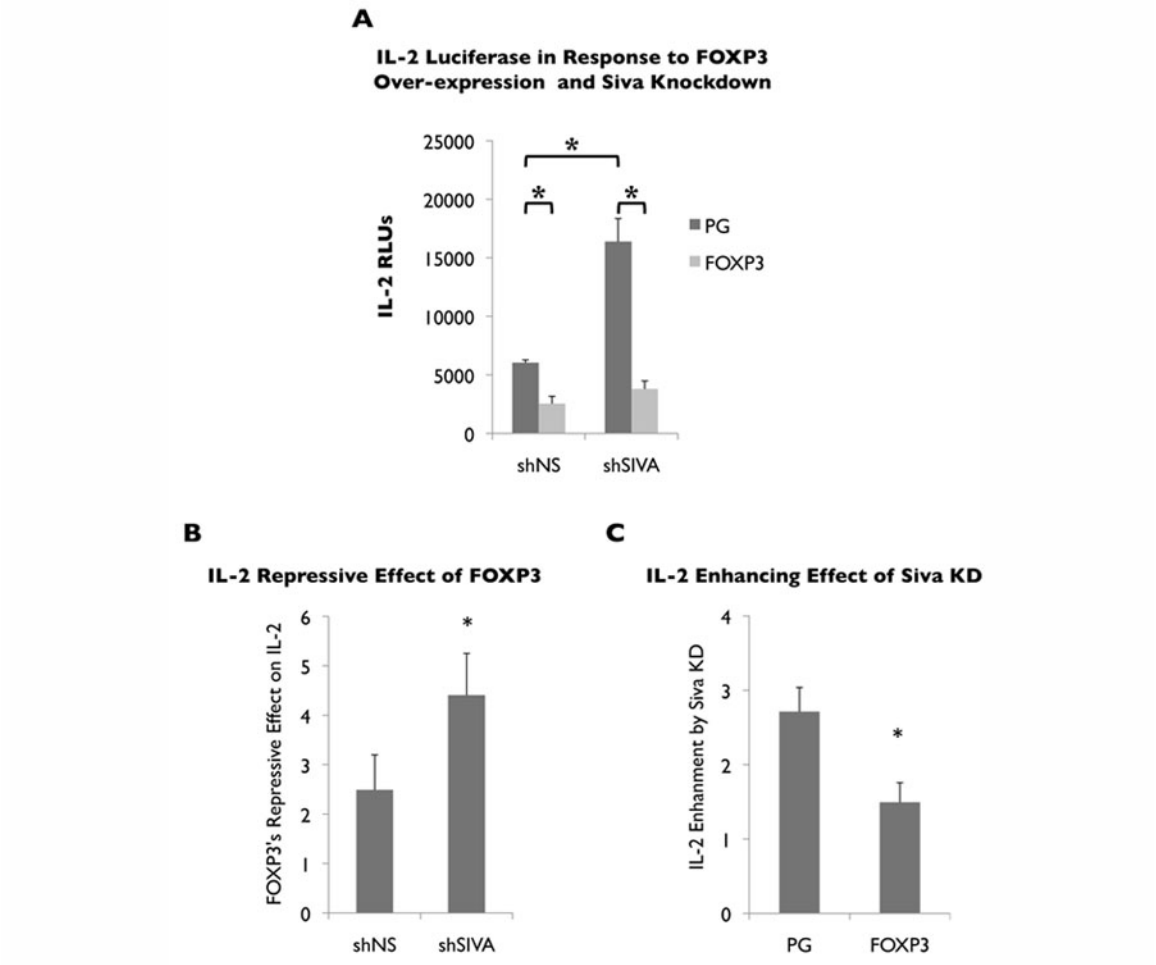


**Figure 2.11: *IL-2* promoter activity in response to *FOXP3* overexpression and *Siva* knockdown (KD).**

*IL-2* promoter activity was evaluated using methods similar to those described for Figures 2.7 and 2.10. Jurkat T cells were transfected with the *IL-2 luciferase* reporter plasmid, a vector containing a negative control hairpin sequence (shNS) or shSIVA and pHSPG or pHSPG-FOXP3.

A. *IL-2*-driven luciferase activity in response to *FOXP3* overexpression and *Siva* KD. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test.
B. *FOXP3's* repressive effect on *IL-2* promoter activity was determined by calculating fold repression in the presence or absence of *Siva* KD. Ex. (shNS:PG)/(shNS:FOXP3) vs. (shSIVA:PG)/(shSIVA:FOXP3). * indicates statistical difference between bars shown based on $p<0.05$ by a two-tailed Student's t-test.
C. *Siva KD's* enhancing effect on *IL-2* promoter activity was determined by calculating fold repression in the presence or absence of *FOXP3*. Ex. (shSIVA:PG)/(shNS:PG) vs. (shSIVA:FOXP3)/(shNS:FOXP3). * indicates statistical difference between bars shown based on $p<0.05$ by a two-tailed Student's t-test.

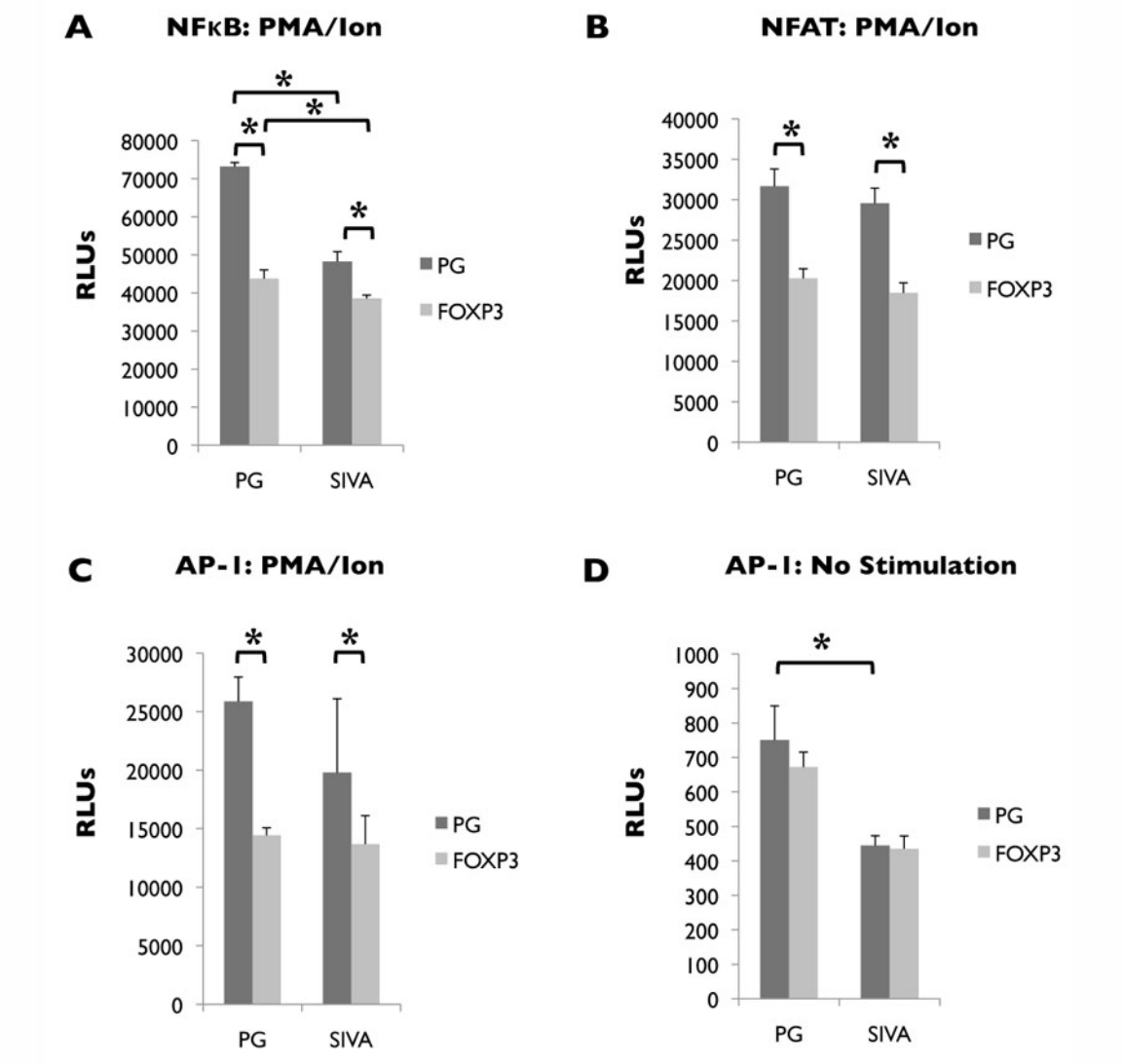


**Figure 2.12: Transcriptional activity of three *IL-2* regulatory elements in response to *FOXP3* and *Siva*.**

Jurkat cells were transfected by electroporation with a luciferase reporter plasmid and plasmid expressing *FOXP3*, *Siva* or the parental vector control, using methods previously described (Figures 2.7, 2.10 and 2.11). Brackets and *s indicate statistical difference between indicated pairs based on $p<0.05$ by a two-tailed Student's t-test. Data shown represents experiments that have been performed twice.

A. Effect of *FOXP3* and *Siva* on NFκB activity in response to PMA/Ion. The reporter was comprised of 3 tandem repeats of the MHC-κB response element (MHC-3κB).
B. Effect of *FOXP3* and *Siva* on NFAT activity in response to P/I. The reporter was comprised of 3 tandem repeats of the *IL-2* promoter NFAT response element.
C. Effect of *FOXP3* and *Siva* on AP-1 activity in response to P/I. The reporter was comprised of 3 tandem repeats of the TPA response element.
D. Effect of *FOXP3* and *Siva* on AP-1 activity in the absence of stimulation.

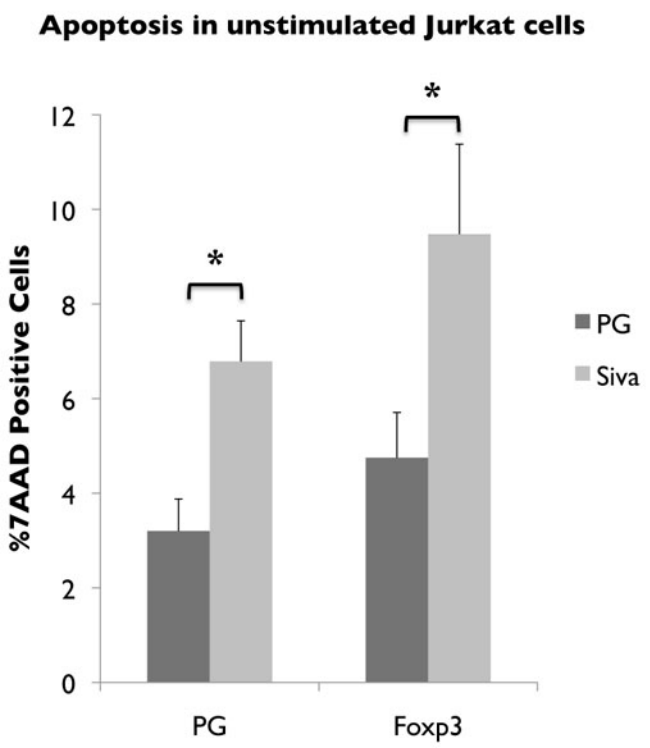


**Figure 2.13: *Siva's* pro-apoptotic effect is not significantly altered by *FOXP3* in unstimulated Jurkat T cells.**
The viability of Jurkat T cells transduced with pHSPG, pHSPG-Siva, and/or pHSPG-FOXP3 (transduction efficiency shown in Figure 2.8A) was determined by staining with 7AAD (marker of late apoptosis). Brackets and *s indicate statistical difference between pairs based on $p<0.05$ by a two-tailed Student's t-test. Data shown represents experiments that have been performed twice.

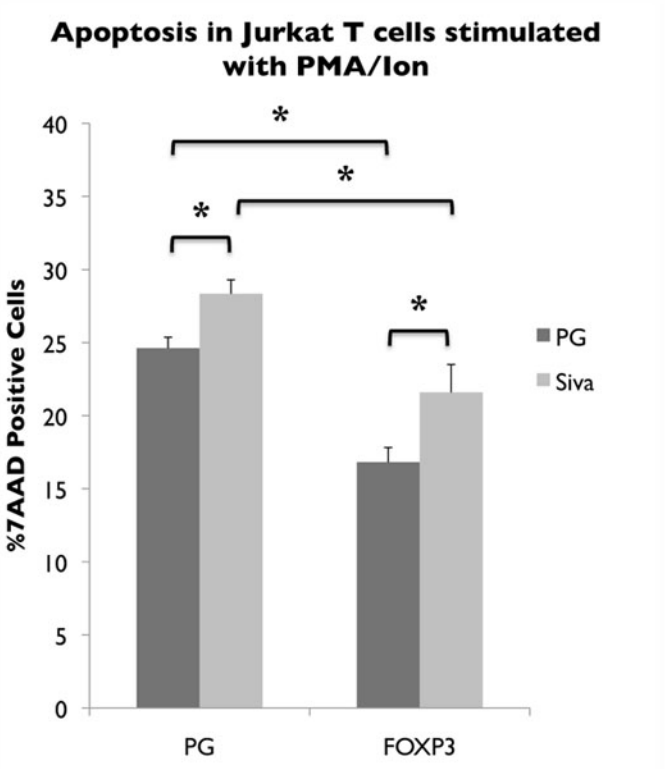


**Figure 2.14: Effects of *Siva* and *FOXP3* on apoptosis in PMA/Ion-treated Jurkat T cells.** Transduced Jurkat T cells shown in Figures 2.8 and S2.13 were activated with PMA/Ion for 18 hours and viability was assessed by 7AAD staining. Brackets and *s indicate statistical difference between designated pairs based on $p<0.05$ by a two-tailed Student's t-test. Data shown represents experiments that have been performed twice.

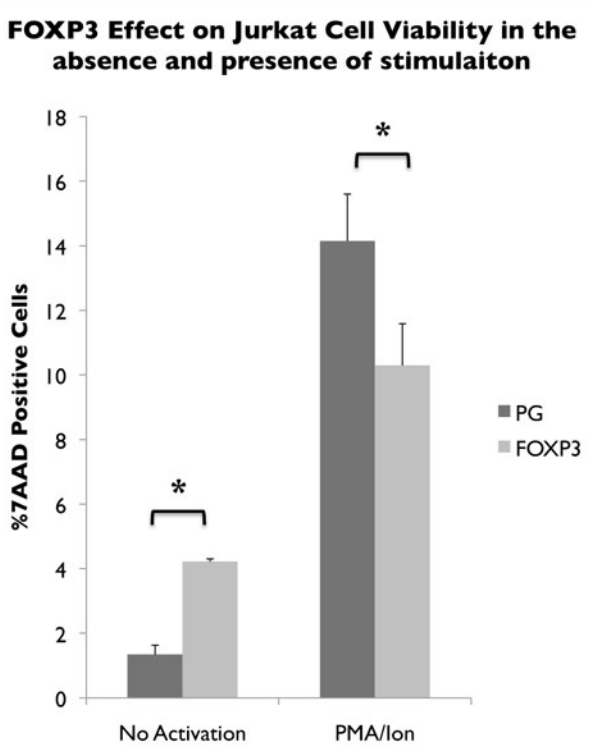


**Figure 2.15: Differential effect of *FOXP3* on Jurkat T cell viability is dependent upon stimulation conditions.**
Jurkat T cells were transduced with pHSPG (PG) or pHSPG-FOXP3 (efficiency >95% for both populations). Transduced cells were plated in 96-well plates at a density of $2x10^5$ cells/well. Cells were left unstimulated or activated with PMA (25 ng/ml) and Ion (1 μM) for ~18 hours. Cell viability was evaluated by staining with 7AAD, a marker of late apoptosis. * indicates $p<0.05$ for two-tailed Student's t-test between PG- and FOXP3-transduced cells. Experiment was performed three times.

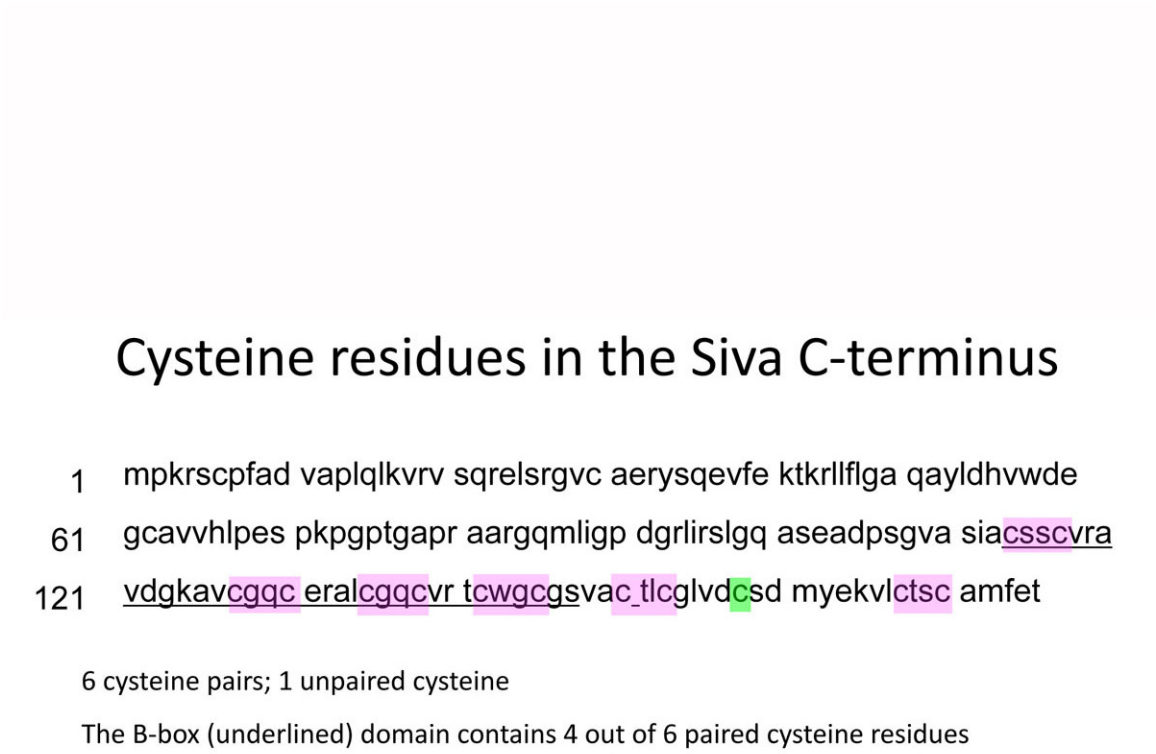


**Figure 2.16: Siva-1 protein sequence highlighting the presence of cysteine residues in the C-terminus.**

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# CHAPTER THREE

## FOXP3 REGULATION OF HER2/NEU IN THYMIC EPITHELIAL CELLS (TECS) AND HER2/NEU FUNCTION IN TECS

ABSTRACT

In this chapter, I will present data pertaining to *Foxp3* regulation of *Her2/neu* in TECs and the effect of Her2/neu signaling on thymopoiesis. Previously, it was reported that Her2/neu is upregulated in TECs from *scurfy* mice, a *Foxp3*-deficient strain. Based on the finding that Herceptin, a Her2/neu-specific monoclonal antibody, partially rescued defective thymopoiesis in *scurfy* mice[1], we hypothesized that Her2/neu signaling could be involved in TEC function. To investigate the effect of Her2/neu on thymopoiesis, we treated fetal thymic organ cultures (FTOC) with a small molecule inhibitor of Her2/neu and EGFR, GW572016 (GW57). GW57 treatment moderately enhanced thymopoiesis in the FTOC.

Also, we investigated the relationship between *Foxp3* and *Her2/neu* expression in TEC lines representative of cortical, medullar, and sub-capsular thymic regions. We observed an inverse relationship between *Foxp3* and *Her2/neu* across three TEC lines. *Foxp3* overexpression repressed *Her2/neu* in two out of three TEC lines tested (cortical 1308 and sub-capsular 427), but not in the TEC line expressing the highest amount of *Foxp3* and lowest *Her2/neu* (medullary 1307).

In summary, data from the TEC lines supports the assertion that *Foxp3* represses *Her2/neu.* Further, inhibition of Her2/neu and EGFR by GW57 positively affected thymopoiesis in some FTOC models. These experiments suggest that improved understanding of how Her2/neu and EGFR contribute to thymopoiesis could eventually lead to improved thymus-targeted therapies that would be beneficial for patients with immunodeficiencies due to HIV-1 infection or chemotherapy.

## INTRODUCTION

A functional thymus is essential for limiting infections and preventing autoimmune disease. Thymus function depends on the differentiation and integrity of thymic epithelial cells (TECs). TECs form a three dimensional reticular structure that houses thymocytes and provides developmental cues that guide T cell lineage commitment, differentiation, proliferation and selection[2,3]. In spite of the important contributions of TECs to thymopoiesis, relatively little is known about TEC biology compared to the T cells that TECs support[4].

In humans, *FOXP3* mutations cause a severe autoimmune disease called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [5-7]. Similarly, the *scurfy* mouse strain carries a *Foxp3* frameshift truncation mutation and affected males succumb to lethal, multi-organ T cell proliferative disease[6,8].

Most investigators attribute the severe autoimmune syndrome in IPEX patients and *Scurfy* mutants to T-cell intrinsic *Foxp3* deficiency. *Foxp3* is required for the development, function, and maintenance of natural regulatory T cells ($nT_{regs}$), a critical $CD4^{pos}$ population that suppresses autoreactive T cells and prevents destructive inflammation. The absence of $T_{regs}$ associated with *Foxp3* deficiency has been proposed as the central causal factor for autoimmunity in mice and humans[9-12].

In addition to the T-cell intrinsic function of *Foxp3,* support for *Foxp3* function in TECs first came from transgenic radiation chimeras. *Scurfy* bone marrow failed to transfer autoimmunity into irradiated SCID (severe combined immunodeficiency) mice, indicating that radioresistant non-bone marrow-derived cells (which may have included TECs) required the *Scurfy* mutation to elicit autoimmunity. However, the later discovery that radiation

induced T cell production in SCID mice compromised interpretation of the *scurfy*/SCID chimeras. The experiments were repeated in *RAG*-/- (recombination activating gene) mice. Again, *scurfy* bone marrow failed to transfer autoimmunity (Reviewed in [13]).

In another set of experiments, the *scurfy* allele (*Foxp3$^{Sf}$*) was crossed on to the *RAG*-/- background and bone marrow transplant experiments demonstrated impaired thymopoiesis in *RAG-/-Foxp3$^{Sf}$* recipients. T-cell depleted bone marrow was transferred from GFP (green fluorescent protein) transgenic mice into sublethally irradiated recipients (*RAG-/-Foxp3$^{wt}$* compared to *RAG-/-Foxp3$^{Sf}$*). Thymocyte distribution and proliferation was measured to evaluate stromal cell function. The distribution of mature single positive (SP, cells single positive for either the CD4 or CD8 coreceptor) thymocytes was normal. In contrast, the earliest stage of thymopoiesis was defective. The immature double negative (DN, marked by the absence of CD4 and CD8 coreceptors) compartment in *RAG-/-Foxp3$^{Sf}$* mice contained fewer proliferative cells based on BrdU (5-bromo-2-deoxyuridine) incorporation compared to *RAG-/-Foxp3$^{wt}$*. *Foxp3* mRNA and protein were detected in TECs by realtime PCR and immunofluorescence, respecitively[1]. These data led to the proposal that T-cell extrinsic properties of *Foxp3* also contribute to autoimmunity in mice and humans[1,13,14].

Following a serendipitous observation that female *Foxp3$^{Sf}$* heterozygotes developed spontaneous mammary carcinomas, Zuo *et al*[15] discovered that *Foxp3* exerted tumor suppressor properties by repressing *Her2/neu*, an oncogene associated with breast cancer[15]. The *Her2/neu*-repressive effect of *Foxp3* in breast epithelium prompted analysis of *Her2/neu* expression in thymic epithelium.

Her2/neu overexpression by TECs was observed in the *scurfy* mice and was proposed as a mechanism by which *Foxp3* contributed to TEC function[1]. Her2/neu (name derived from human epidermal growth factor receptor-2/neu oncogene) is an orphan receptor from the receptor tyrosine kinase, epidermal growth factor receptor (EGFR) family. As a family, EGFRs regulate diverse cell fate programs including differentiation and proliferation. Her2/neu overexpression is associated with malignancy. Herceptin is a monoclonal antibody that inhibits Her2/neu signaling and was developed to treat Her2/neu$^{pos}$ cancers[16]. Herceptin treatment partially rescues defective DN thymocyte proliferation in *scurfy* mice.

Thus, at the start of my project, data existed in support of the following premises:

1. TECs express *Foxp3* and *Foxp3* contributes to TEC function.
2. *Foxp3* represses Her2/neu protein expression.
3. Her2/neu antagonism improved defective thymopoiesis in *Foxp3*$^{Sf}$ mice.

During the course of research, *Foxp3* expression and function in TECs was challenged by evidence from three different transgenic *Foxp3* promoter-driven *GFP* reporter mice[10,11] and by the discovery of radiation-resistant *Foxp3*$^{pos}$ cells[12]. Regardless of the controversy over *Foxp3* function in TECs, the negative regulatory effect of *Foxp3* on *Her2/neu* has not been contested and the effects of Her2/neu on thymopoiesis have yet to be fully elucidated[17,18].

To study the effect of *Foxp3* overexpression on *Her2/neu* expression, we used three TEC lines associated with different cortical, medullary, and subcapsular thymic regions[19]. We observed an inverse relationship between *Foxp3* and *Her2/neu* across the three TEC

lines. Ectopic expression of *Foxp3* by retroviral (RV) transduction yielded *Her2/neu* repression in two TEC lines, but not in the TEC line expressing the highest level of *Foxp3*.

Next, because epigenetic modifications are associated with gene regulation, we tested the effect of two chromatin remodeling inhibitors on *Her2/neu* expression: Trichostatin A (TSA) and 5-azacytidine (AZA). TSA suppresses histone deacetylase (HDAC) activity and AZA inhibits DNA methylation. Epigenetic transcriptional regulation is poorly understood. Histone acetylation is associated with transcriptionally active chromatin and reduced histone acetylation is associated with inactive chromatin. Conversely, methylated DNA is associated with transcriptional repression[20].

In order to investigate Her2/neu function in thymopoiesis, we used two types of fetal thymic organ culture (FTOC) systems. In the simplest FTOC model, unmanipulated fetal thymic lobes were cultured on a membrane *in vitro.* The simple FTOC model does not segregate TECs from thymocytes. The hanging drop FTOC involves more manipulations, but permits modification of TECs separate from thymocytes[21]. Therefore, the hanging drop FTOC is an ideal system for studying TEC-intrinsic factors.

Research in the field of oncogenic inhibitor drugs has led to the creation of compounds that specifically target known transformation signaling pathways. The small molecule, GW572016 (referred to as GW57) was developed by Glaxo Smith Kline, formerly Glaxo Wellcome, to inhibit EGFR and Her2/neu mediated proliferation[22-24].

We tested the effect of dual Her2/neu and EGFR inhibition by applying GW57 to thymic lobes in the standard FTOC and hanging drop FTOC system. Inhibition of

EGFP/Her2/neu moderately improved thymopoiesis in the FTOC system under some conditions.

## METHODS AND MATERIALS

### *Cell lines and culture conditions*

The TEC cell lines 427.1, 1307.6.1.11, and 1308.1.6 were originally derived from SV40 T-antigen transgenic (SV40T-Tg) mice[19].

Line 427.1 was derived by directly culturing dissociated hyperplastic thymus tissue from SV40T-Tg mice. Routine trypsin treatment removed contaminating fibroblasts. The dominant epithelial cell line that remained after repeated rounds of trypsanization was derived by single-cell limiting dilution. To derive parental TEC lines 1307 and 1308, hyperplastic thymic tissue was taken from SV40T-Tg mice, dissociated, and serially passed in nude mice. The thymus cells were either injected into the peritoneal cavity or surgically placed under the kidney capsule. Tumors formed, which were serially transferred into nude mice two more times. Abdominal tumors were taken from the third recipient, dissociated and cultured. Limiting dilution was performed to isolate parental clones. Based on morphology and ultrastructural features, the clones were proposed to represent distinct thymic epithelial populations[19].

427.1 cells were described as thymic subcapsular cortex or thymic nurse cells; 1308.1 were thought to be deep cortex or cortical reticular cells; and 1307.6.1.11 shared characteristics with cells of medullar origin. TEC lines 427.1, 1307.6.1.11, and 1308.1.6 will be referred to as 427, 1307, and 1308, respectively.

Adherent TEC lines, and 293T cells were cultured at 37° in 5%$CO_2$ in DMEM-10% FBS supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Trichostatin A and 5'-Azacytidine were purchased from Sigma. GW572016 (GW57) was a gift from Shelton Earp[24].

*Gene expression measurements by PCR*

The Qiagen RNeasy isolation kit was used to isolate mRNA from all cell lines. Reverse transcription (RT) reactions were performed with random primers and M-MLV reverse transcriptase in the presence of RNase inhibitor. Quantitative Taq-Man PCR was performed using primers from Applied Biosystesms. Semi-quantitative RT PCR was performed by serially diluting the cDNA template ten fold. The following primer sequences were used in semi-quantitative RT PCRs:

*Her2/neu For,* 5'-GCTGCTGGACATTGATGAGA-3'

*Her2/neu Rev* 5'-GGGATCCCATCGTAAGGTTT-3'

*HPRT For,* 5'-CAGGCCAGACTTTG-3'

*HPRT Rev,* 5'-TTGCGCTCATCTTAGGCTTT-3'

*Retrovirus production and transduction*

The pHSPG and pHSPG-mFoxp3 retrovirus (RV) used to transduce TEC lines was prepared in 293T cells by the calcium phosphate transfection method, which has been described previously[21,25]. Briefly, DNA complexes were prepared by mixing plasmids for the RV vector, VSV-G, and gag/pol with HBSS and calcium chloride. Adherent 293T cells were treated with chloroquine thirty minutes prior to the addition of DNA. Supernatants were collected at 48 and 72 hours.

*Western blot, antibodies, and 293T cell transfections*

For protein analysis by western blot, cell pellets were washed in PBS and stored at -20°. In this report, all cell pellets were lysed in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, and 50 mM NaF). Protein concentrations were determined using the Pierce BCA kit and 50 μg lysate was mixed with 2X SDS loading dye. Samples were boiled and separated by electrophoresis on a 10% Bis-Tris NuPage gel. Gel contents were transferred to a PVDF membrane (Amersham Hybond-P).

Membrane blocking (~30 minutes, room temperature) and all subsequent antibody incubation steps were performed in PBS/0.2%Tween-20/5% milk. Primary antibody incubations were performed overnight at 4°, followed by four ~7 minute washes in PBS/0.2%Tween-20. Her2/neu expression was detected with Rabbit αHer2/neu RB-103-P from NeoMarkers (1:1000). The polyclonal Foxp3 serum (diluted 1:2000) used in this report was generated by immunizing rabbits with a Foxp3 N-terminal peptide (CLLGTRGSGGPFQGRDLRSGAH)[26]. Mouse monoclonal antibodies against β-actin (Sigma, AC-74, 1:10,000) and Myc (9E10 hybridoma supernatant kindly provided by Dr. Yue Xiong, 1:500) were also used. Goat anti-mouse-HRP or donkey anti-rabbit-HRP were used to detect mouse- and rabbit-derived primary antibodies, respectively (diluted 1:10,000).

293T cell transfections were performed using Qiagen Effectene, according to the manufacturer's intructions. 293T cells were harvested at 48-hours post-transfection.

*Generation of mouse Foxp3 short hairpin RNA targeting construct*

The *mFoxp3* gene sequence (NM_054039) was entered into the Dharmacon siDESIGN® Center online tool to obtain short sequences predicted to have interference

activity. A target sequence was selected because it spanned a region of incomplete homology between the *mFoxp3* and *hFOXP3* DNA sequence (3 out of 15 base pair differences). Oligos encoding the sense, the stem-loop, and the anti-sense sequences were designed to be subcloned into the pLL5.0 lentiviral vector[27], which was kindly provided by Dr. Jim Bear (5'-TGGACCGTAGATGAATTTGGTTCAAGAGATCAAATTCATCT ACGGTCCTTTTTTC-3', underlined amino acids indicate location of the hairpin loop). PAGE purified oligos with 5' phosphates were purchased from Operon.

*Fetal thymic organ culture (FTOC) and analysis*

Variations on the fetal thymic organ culture (FTOC) system have been reported previously by numerous groups, including ourselves[21,28]. Time of pregnancy was recorded in order to collect fetal thymic lobes on embryonic day 14 (E14). During the dissection procedure, lobes were collected in PBS on ice. Thymic lobes were cultured on Millipore membranes in mFTOC media (RPMI-10% FBS supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50M β-mercaptoethanol, 1% nonessential amino acid, 10 mM Hepes, and 1 mM sodium pyruvate solution). The culture media was changed every two days. GW57 or DMSO vehicle was added to media where indicated.

Fetal liver (FL) cells were also collected from E14 pups. Mouse FL cells were cultured in mFTOC media supplemented with hIL-11 (10 ng/ml), mIL-6 (50 ng/ml), and mSCF (stem cell factor, 50 ng/ml). All cytokines were purchased from Peprotech.

For the hanging drop experiments (outlined in Figure 3.9), lobes were depleted of dividing cells by 2- deoxyguanosine (2dG, 1.35 mM) treatement for 5 days. Depleted lobes were washed in PBS and half of the lobes were exposed to γ-irradiation (500R in

experiments reported here; Lobes also survived 750R). To seed lobes, E14 FL cells were seeded to a density of $10^5$ cells/40 µl mFTOC media/well in Terasaki plates. A single lobe was carefully added to each well. Plates were inverted to allow migration of progenitor cells into the lobe. After 48-hours, lobes were washed in PBS/2% FBS and placed back on membranes. As described above, media was changed every two days and GW57 or vehicle control was applied where indicated.

At the end of three weeks, lobes were transferred to 500 µl PBS/2% FBS in microcentrifuge tubes. A plastic pestel (Bellco Glass, Inc, Cat# 1980-45015) was used to manually release thymocytes from the thymic capsule. Cells were counted in Gauva ViaCount reagent on a Gauva EasyCyte. Cells were stained with CD4-FITC and CD8-PERCP and analyzed on a Becton-Dickson FACScan.

## RESULTS

### *Correlation between endogenous Foxp3 and Her2/neu in TEC Lines*

Before performing any genetic manipulations with the TEC lines, we evaluated *Foxp3* and *Her2/neu* gene expression by quantitative realtime PCR. Judged against total adult thymocytes, *Foxp3* expression in all three TEC lines was very low (Figure 3.1A). Comparison between the three TEC lines indicated that *Foxp3* was highest in the medullary-like 1307 line and lowest in the subcapsular-like 427 line. Next, we measured *Her2/neu* expression. We found *Her2/neu* was highest in 427 TECs and lowest in 1308 TECs. Thus, an inverse relationship exists between *Foxp3* and *Her2/neu* across the three TEC lines (Figure 3.1B). Although this relationship is consistent with a repressive effect of *Foxp3* on *Her2/neu, Foxp3*-independent factors could account for the observation.

### *Effect of ectopic Foxp3 expression on Her2/neu in TECs*

Next, we tested the hypothesis that *Foxp3* affects *Her2/neu* expression in TEC lines. Each line was transduced with pHSPG control or pHSPG-mFoxp3 retrovirus (RV). Transduced cells were collected three days after transduction and saved for mRNA or protein analysis.

Her2/neu protein was repressed by *Foxp3* expression in the cortical TEC line, 1308 (Figure 3.2). Transfected 293T cells were used as a western blot staining control. It is worth noting that Her2/neu expression did not change in 293T cells transfected with *Foxp3*. *Foxp3* overexpression repressed *Her2/neu* mRNA in thymic subcapsular-like 427 cells, but not in medullary-like 1307 cells, which already have low Her2/neu expression (Figure 3.3). Out of the three TEC lines, 1307 cells expressed the highest level of *Foxp3*. The absence of a

*Foxp3*-mediated repressive effect on *Her2/neu* in 1307 cells might be due to sufficient levels of endogenous *Foxp3* already present, which have saturated the repressive effect. In order to test the hypothesis that *Foxp3* in 1307 cells affected *Her2/neu* expression, we planned to knockdown *Foxp3* expression in 1307 cells with short hairpin RNA (shRNA).

*Mouse Foxp3 knockdown by shRNA*

The *sh-mFoxp3* sequence was subcloned into the pLL5.0 lentiviral (LV) vector, under control of the U6 promoter (Figure 3.4A)[27]. To test the specificity and efficiency of pLL5.0-sh-mFoxp3, we co-transfected 293T cells with pLL5.0-sh-mFoxp3 or pLL5.0-NS (not specific) and either pcDNA-Myc/hFOXP3 or pcDNA-Myc/mFoxp3. Western blot analysis clearly shows that pLL5.0-sh-mFoxp3 reduces mouse Foxp3, but not human FOXP3 protein (Figure 3.4B)

*Chromatin modifying inhibitors*

While the histone code hypothesis predicts that inhibition of histone deacetylation and DNA methylation open up chromatin and relieve transcriptional repression for most genes, individual genes are differentially sensitive to epigenetic remodeling mechanisms. Therefore, we tested the effects of chromatin remodeling inhibitors TSA and AZA on *Her2/neu* and *Foxp3* expression in 427 cells. Both TSA and AZA repressed *Her2/neu* in 427 cells (Figure 3.5A&B). In contrast, *Foxp3* expression was enhanced by both TSA and AZA (Figure 3.5C&D).

*Effect of Her2/neu/EGFR inhibition on thymopoiesis in FTOC*

Her2/neu and EGFR share similar tyrosine kinase signaling domains in their cytoplasmic tails. The small molecule GW57 efficiently blocks downstream MAPK (mitogen activated protein kinase) signaling and cell proliferation through inhibiting the kinase activity of both Her2/neu and EGFR[22-24]. Based on observations that *Her2/neu* was elevated in the *scurfy* mouse and that Herceptin treatment partially rescued deficient thymopoiesis[1], we hypothesized that inhibition of Her2/neu with GW57 would enhance thymopoiesis in the FTOC. We took E14 thymic lobes and cultured the lobes on membranes in the presence of DMSO drug vehicle or titrated doses of GW57. At the end of the experiment, we harvested the lobes and analyzed thymocyte output. Dual inhibition of Her2/neu/EGFR signaling moderately enhanced thymocyte number with statistical significance at 5μM and 25μM GW57, but not 1μM (Figure 3.6). The increase in thymocyte output corresponded to decreased number of immature double negative (DN, $CD4^{Neg}CD8^{Neg}$) cells and increased number of double positive cells (DP, $CD4^{Pos}CD8^{Pos}$)(Figure 3.7B&C). At 25μM GW57, CD4 single positive (SP) cell number per lobe was increased compared to the DMSO control (Figure 3.7D). GW57 did not affect CD8 SP cell number per lobe (Figure 3.7E).

*Effect of Her2/neu/EGFR inhibition on thymopoiesis in irradiated FTOCs*

One reason the GW57 treatment in FTOC did not show a greater effect on thymopoiesis could be due to relatively low expression level of Her2/neu in fetal thymic epithelium. *Her2/neu* sensitizes breast epithelial cells to radiation[29] and we observed increased Her2/neu in the thymus in response to whole body irradiation (Figure 3.8). Given *Her2/neu's* radiosensitizing role and that the effect of GW57 on thymopoiesis was small

(~25% increase in thymocyte number), we hypothesized that GW57 might enhance thymopoiesis to a greater degree following irradiation. To test the effect of irradiation and GW57 on thymopoiesis, we used the hanging drop FTOC system, which is outlined in Figure 3.9.

Irradiation of the thymic stroma caused a two-fold decrease in thymocyte output, but the effect was not statistically significant (Figure 3.10). In non-irradiated lobes (Figure 3.11A), 5μM GW57 treatment caused a small, but statistically significant decrease in thymocyte number. 10μM GW57 had no effect on thymocyte number in non-irradiated lobes. In contrast, 5μM GW57 enhanced thymocyte output in irradiated lobes (Figure 3.11B). 10μM GW57 also enhanced thymocyte output in irradiated lobes, but the effect was not statistically significant. No preferential effect on thymocyte distribution was observed (Figure 3.12). Thus, dual inhibition of Her2/neu and EGFR moderately enhanced thymopoiesis in irradiated lobes, but not in non-irradiated lobes.

## DISCUSSION

This project generated support for the hypothesis that *Foxp3* represses *Her2/neu* gene expression in TECs. Further, we demonstrated that HDAC and DNA methyltransferase inhibition repressed *Her2/neu,* which is consistent with the observed effects of HDAC inhibition on *Her2/neu* in breast cancer epithelial cells[30,31]. Finally, inhibition of Her2/neu (but also EGFR) signaling by GW57 treatment moderately enhanced thymopoiesis in some fetal thymic organ culture (FTOC) models.

In the hanging drop FTOC model, 5 μM GW57 enhanced thymic output in irradiated lobes, but repressed thymic output in the absence of radiation (Figure 3.11). It is difficult to draw meaningful conclusions from this data because EGFR and Her2/neu are both inhibited by GW57 and the expression kinetics of each in response to radiation in the FTOC model was not measured. Therefore, more information regarding the kinetics and function of EGFR and Her2/neu in TECs is needed. Experiments evaluating the effects of individual EGFR and Her2/neu inhibtors in the FTOC model would greatly improve upon the current understanding of how each of these signaling molecules contributes to thymopoiesis[29]. Such experiments could lead to improved thymopoietic enhancement therapies, which might help treat immunodeficiency in HIV-1 infected individuals and patients receiving chemotherapy[32].

### *Her2/neu and thymus biology*

The effect of dysregulated Her2/neu expression on TEC biology is still an open question. *Her2/neu* overexpression in TECs is associated with poor thymic function. Kertain-5 is found in skin epithelium and in TECs, where it is associated with the medulla and the cotico-medullary junction[33]. Transgenic mice carrying bovine *keratin-5*-driven *Her2/neu*

overexpress Her2/neu in medullary TECs. *Keratin-5-Her2/neu* transgenic mice had thymic abnormalities including increased thymocyte apoptosis and loss of thymic cortico-medullary organization[18]. Another group used Cre/lox technology to delete *Stat3* in Keratin-5 expressing TECs. TEC specific *Stat3* deletion caused thymic hypoplasia, impaired thymocyte regeneration after irradiation and increased *Her2/neu* expression[17]. Taken together, these results show an association between excessive *Her2/neu* expression and impaired TEC function.

Beyond the negative effect of *Her2/neu* overexpression in TECs, little is known about *Her2/neu* function in TEC biology. In breast cancer cells, *Her2/neu* promotes cell proliferation[16,29]. One reason *Her2/neu* negatively affects thymopoiesis could be due to enhanced proliferation of TECs that are immature, or otherwise impaired in their ability to support thymopoiesis by some, as yet, unidentified mechanism.

*Controversy surrounding Foxp3 expression in TECs*

As mentioned previously, TEC expression of *Foxp3* has become a topic of controversy. Data from three transgenic mice refute *Foxp3* function in TECs.

The GFP/Foxp3 fusion protein could not be detected in TECs isolated from *Foxp3*$^{GFP}$ reporter mice[10]. Liston and colleagues analyzed CD45$^{neg}$ TECs from *Foxp3*$^{GFP}$ reporter mice by flow cytometry. All CD45$^{neg}$ cells were negative for GFP, indicating the absence of Foxp3 protein from TECs[11].

Liston *et al*[11] suggested that the colocalization between Foxp3 protein and the cortical TEC marker keratin-8 detected by Chang *el al*[1] might have been wrongly interpreted. They suggested two reasons that Foxp3 might have appeared to be contained within TECs. First,

TECs and developing thymocytes form close associations. Second, high immufluorescence background staining might have obscured results. Observations of Foxp3 in thymic nurse cells (TNCs) support Liston's suggestion. TNCs are a specialized cell type that closely interacts with immature thymocytes in the thymic cortex. Immunofluorescence staining plainly showed Foxp3$^{pos}$ cells contained within TNC/thymocyte complexes[34].

Cre/lox elimination of *Foxp3* expression in *CD4* and *FoxN1* expressing cells provide further evidence against a TEC-intrinsic function for *Foxp3.* CD4$^{pos}$ cell-specific ablation of *Foxp3* in CD4-Cre *Foxp3$^{lox}$* mice recapitulated the lymphoproliferative symptoms seen in *Foxp3-/-* mice[10]. *FoxN1*-specific ablation of *Foxp3* from TECs did not cause autoimmunity[11].

In contrast to data that challenges *Foxp3* expression and function in epithelial cells, *Foxp3* protein was detected in mammary, lung and prostate epithelium of *Rag2-/-* mice, but not in *Rag2-/-Foxp3$^{Sf}$* mice[14]. Also, abundant evidence demonstrates loss of *FOXP3* tumor suppressor function in human mammary carcinomas[15,35]. Somatic mutations and deletions of *FOXP3* were apparent in multiple human breast cancer tissue samples and cell lines[15]. Experiments showing that *FOXP3* expression is turned on by *p53* during the DNA damage response could explain the presence of Foxp3 in epithelial tissues[36]. Neither *p53*- nor DNA damage-induced *Foxp3* investigations have yet been reported for TECs. Interestingly, inhibition of *p53* is a strategy that is currently being evaluated in mice as a means of protecting TECs during chemoradiotherapy and bone marrow transplant[37,38]. Successful rescue of TEC function by *p53* inhibition begs more questions as to the relationship between *p53, Foxp3, Her2/neu,* and TEC biology.

Finally, age-dependent *Foxp3* function in TECs is another possible mechanism that could explain the lack of autoimmunity in *FoxN1*-Cre *Foxp3*$^{lox}$ mice. Female *scurfy* carriers up to one year old are healthy, but retired female breeders heterozygous for *Foxp3*$^{Sf}$ formed spontaneous mammary tumors sometime within two years of age[15]. Taken together, these data suggest that the tumor suppressor function of *Foxp3* is needed between one and two years of age in breast epithelial cells. If similar mechanisms regulate *Foxp3* across epithelial cells, then TEC function in *FoxN1*-Cre *Foxp3*$^{lox}$ mice needs to be observed past age one, but Liston's analysis may not have extended longer than four months[11].

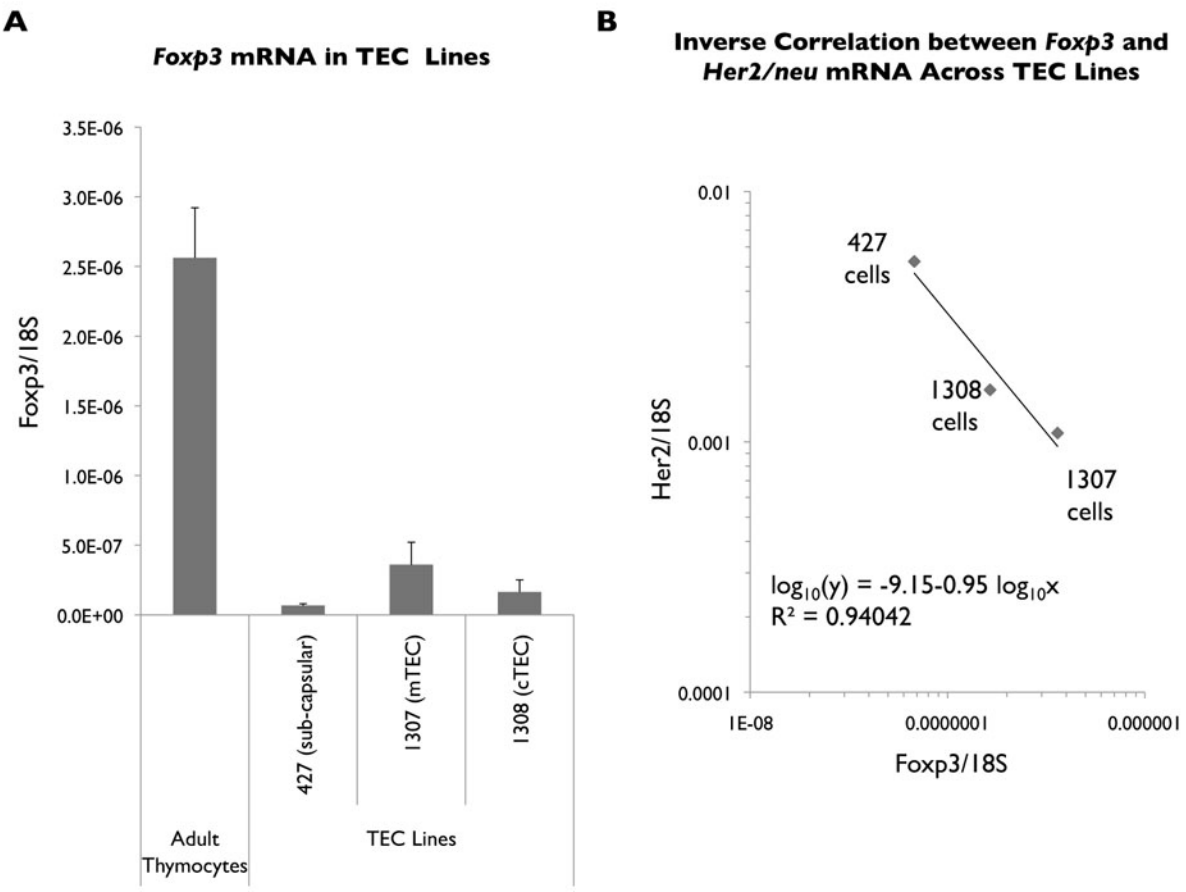


**Figure 3.1: Foxp3 and Her2/neu expression in TEC lines.**
The TEC lines were grown under routine culture conditions and $5x10^4$ cells were collected at time of passage (~80-90% confluency). $5x10^5$ thymoctes were freshly isolated from an eight week old C57/BL6 mouse. Cell pellets were washed in PBS, immediately lysed in Qiagen RLT buffer and stored at -80°. mRNA was isolated by using a Qiagen RNeasy kit, according the manufacturer's instructions. One third of the volume of eluted mRNA (10 µl out of 30 µl) was used to prepare cDNA. Reverse transcription (RT) reactions were performed with random decamer primers, dNTPs, and M-MLV reverse transcriptase in the presence of RNase inhibitor (total volume 20 µl). $1/10^{th}$ of the RT reaction was used in PCRs for *Foxp3* and *Her2/neu*. cDNA was diluted 1000-fold for *18S* PCRs. All genes were measured using TaqMan primers from Applied Biosystems. Relative quantities were derived from $C_T$ values using a standard curve generated by measuring *18S* in serially diluted cDNA from adult thymocytes. Normalization over *18S* accommodated for differences in mRNA input. This experiment was performed twice.

A. *Foxp3* mRNA was detected in TEC lines by realtime PCR and expression was normalized against *18S.*
B. *Her2/neu/18S* was plotted against *Foxp3/18S.* A power regression analysis ($\log_{10}(y)= m + b \log_{10}(x)$, where *m*=slope and *b*=y intercept) was performed to assess the relationship between *Her2/neu* and *Foxp3* across TEC lines.

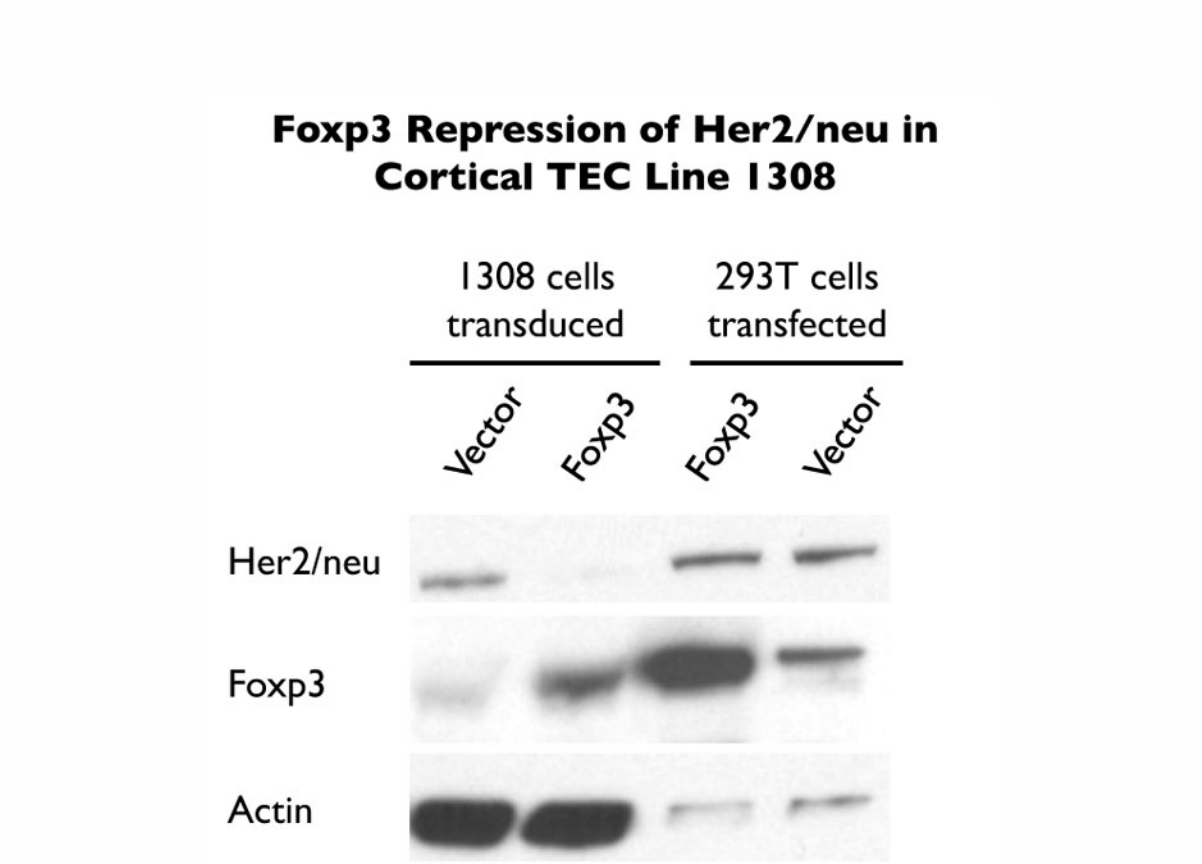


**Figure 3.2: Western blot for Her2/neu and Foxp3 protein in transduced cortical TEC-like 1308 cells.**
The cortical TEC-like line, 1308, was transduced with pHSPG (vector) and pHSPG-Foxp3 retrovirus (RV). Transduced cells were grown under routine culture conditions and GFP expression was measured to determine transduction efficiency. Vector- and pHSPG-Foxp3-transduced cells were 71% and 80% $GFP^{pos}$, respectively. $2x10^6$ cells were collected at time of passage (~80-90% confluency). 293T cells were transfected with plasmids for pHSPG (vector) and pHSPG-Foxp3 using the Qiagen Effectene protocol. Routine western blot procedures are described in Methods and Materials, *Western blot and antibodies*. Her2/neu was detected with a rabbit polyclonal antibody from NeoMarkers (Rb-103-P, diluted 1:1000). The membrane was stripped and reprobed to detect Foxp3 using rabbit α-Foxp3 serum diluted 1:2000. The membrane was stripped and reprobed again with a mouse monoclonal antibody against β-actin (Sigma, AC-74, 1:10,000). This experiment was performed twice.

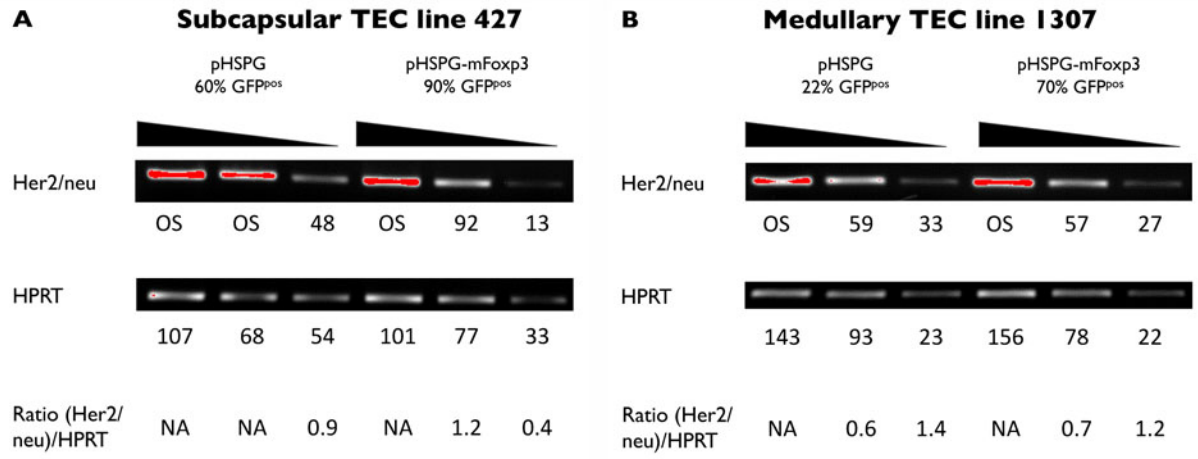


**Figure 3.3: Effect of *Foxp3* on *Her2/neu* expression in 427 and 1307 TEC lines.**
Sub-capsular 427 TECs and medullary 1307 TECs were transduced with pHSPG or pHSPG-Foxp3 retrovirus (RV). Transduction efficiency was determined based on GFP expression and is listed above. mRNA was isolated and used to make cDNA as described in Figure 3.1. $1/10^{th}$ of the cDNA reaction was serially diluted 10-fold, three times. The *Her2/neu* PCRs contained $1/10^{th}$, $1/100^{th}$, and $1/1{,}000^{th}$ of the cDNA reaction and the *HPRT* PCRs contained $1/100^{th}$, $1/1{,}000^{th}$, and $1/10{,}000^{th}$ of the cDNA reaction. $1/6^{th}$ of each PCR was run out on a 1.5% agarose gel. Relative band densities were determined using Image Quant software (OS, over saturated, NA, not applicable). Band density was normalized against a background value for each individual gel. The relative amount of *Her2/neu* expression in each sample is determined by taking the ratio of *Her2/neu* over *HPRT*. This experiment was performed twice.

A. *Foxp3* overexpression with pHSPG RV repressed *Her2/neu* gene expression in subcapsular 427 TECs.
B. *Foxp3* overexpression with pHSPG RV had no effect on *Her2/neu* gene expression in medullary 1307 TECs.

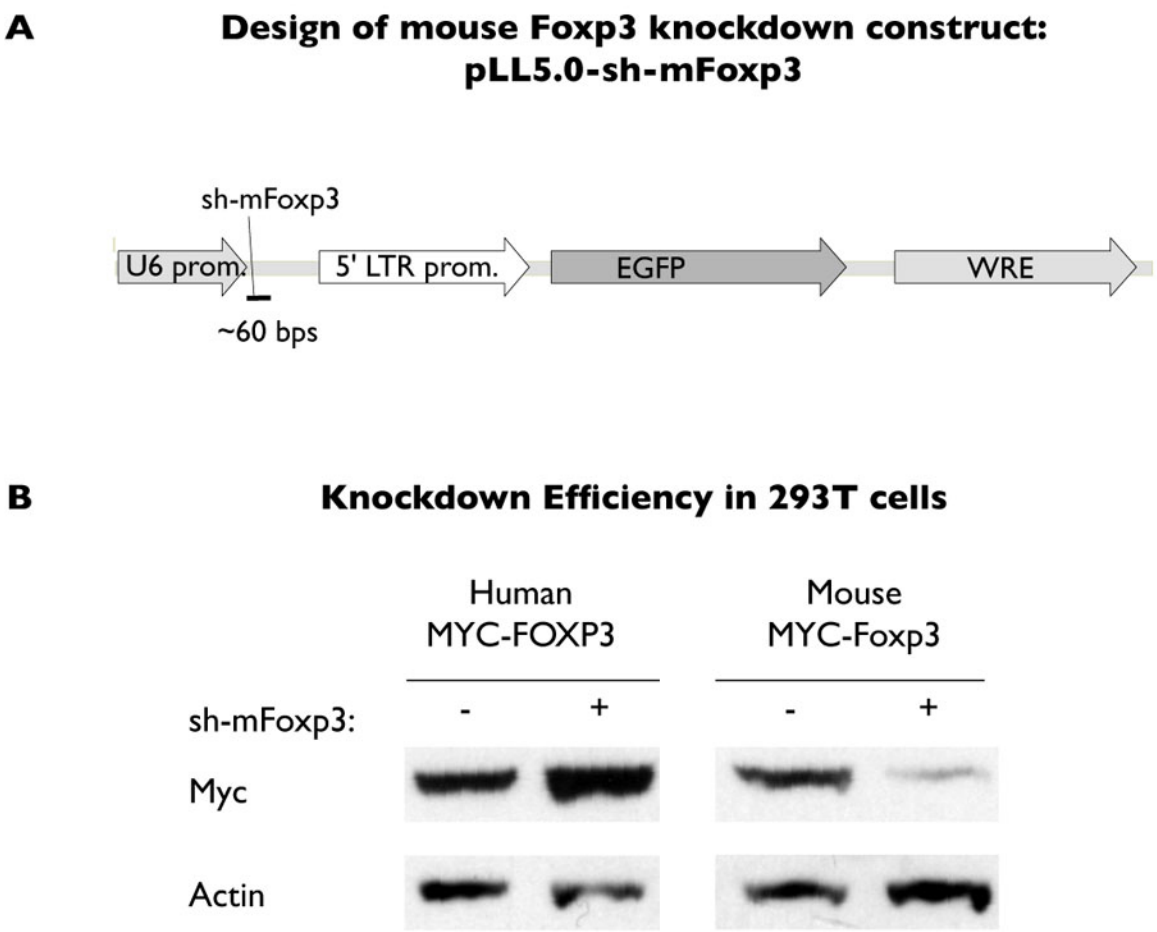


**Figure 3.4: pLL5.0-sh-mFoxp3 design and mouse Foxp3 knockdown.**
The pLL5.0-sh.mFoxp3 vector was designed to selectively knockdown (KD) mouse Foxp3 and not affect human FOXP3 expression.

A. Diagram of pLL5.0-sh-mFoxp3 lentiviral vector. The *sh-mFoxp3* target sequence was selected using siDESIGN® Center, an online siRNA design tool created by Dharmacon. For further details, see Methods and Materials, *Generation of mouse Foxp3 short hairpin RNA construct.*

B. Western blot showing efficient and specific KD of Myc/mFoxp3 protein and resistance of human FOXP3 to KD. 293T cells were co-transfected with pLL5.0-sh-mFoxp3 or pLL5.0-NS (not specific) and either pcDNA-Myc/hFOXP3 or pcDNA-Myc/mFoxp3. This experiment was performed once.

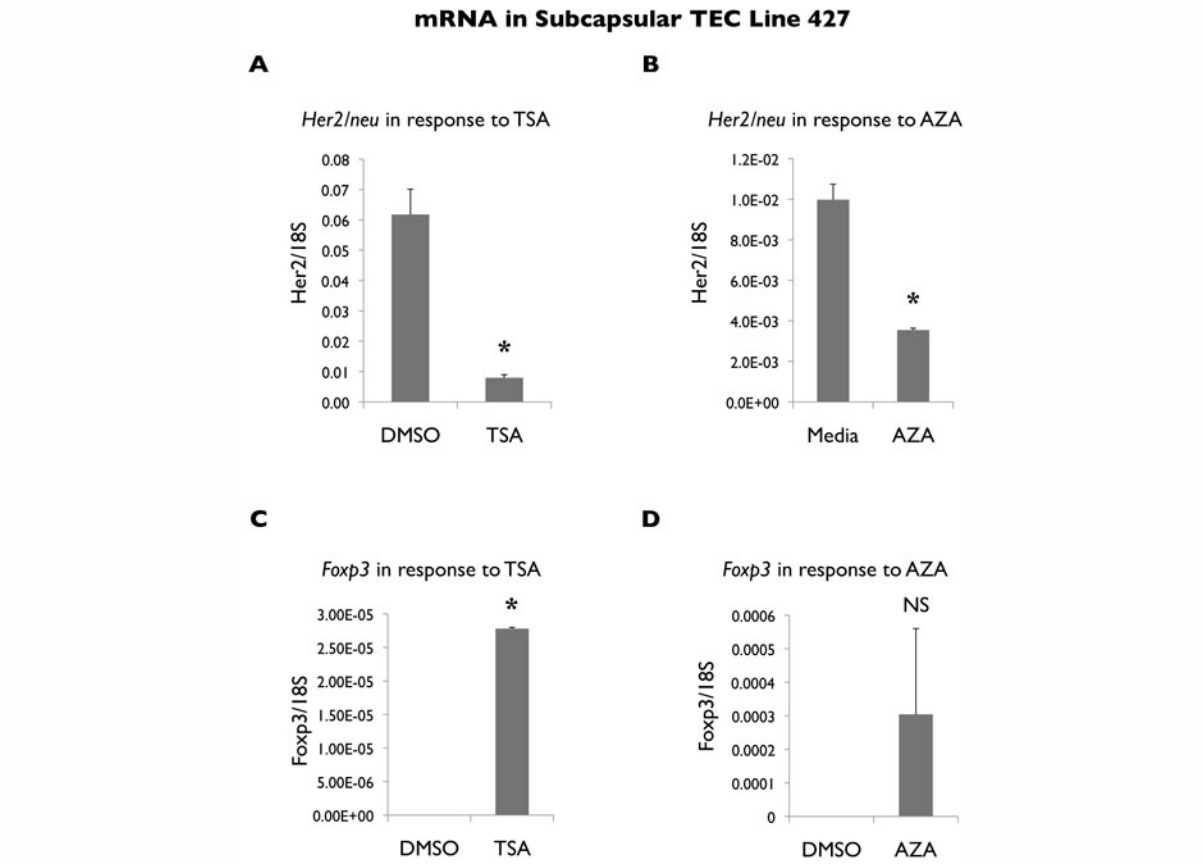


**Figure 3.5: Chromatin remodeling inhibitors repress *Her2/neu* and enhance *Foxp3* expression in subcapsular-like TEC line 427.**
Subcapsular-like TEC 427 cells were plated at a density of $3x10^5$ cells/well in 6-well plates. The next day, adherent cells were treated with chromatin remodeling inhibitors: TSA (1 μM), AZA (1 μM), or drug vehicle, DMSO. After 24 hours, cell pellets were collected and washed with PBS. mRNA was prepared and analyzed as has been described in Figure 3.1. * indicates $p<0.05$ for two-tailed Student's t-test for drug-treated cells compared to DMSO controls. These experiments were performed twice and data shown is representative.

A. AZA treatment represses *Her2/neu* expression in 427 cells.
B. TSA treatment represses *Her2/neu* expression in 427 cells.
C. AZA treatment enhances *Foxp3* expression in 427 cells.
D. TSA treatment enhances *Foxp3* expression in 427 cells.

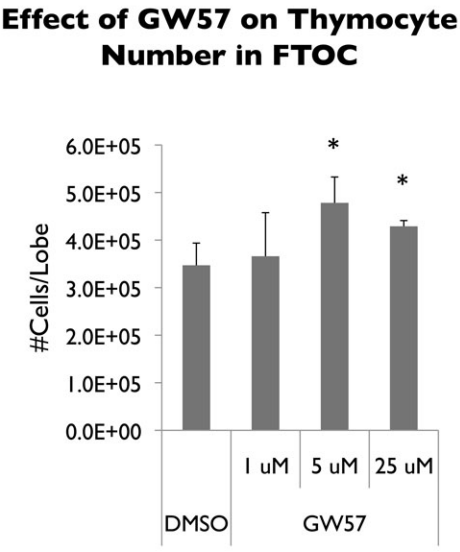


**Figure 3.6: Effect of GW57 on thymocyte number in unmanipulated E14 thymic lobes.** Thymic lobes were removed from E14 pups and cultured for three weeks in mFTOC media with DMSO vehicle or increasing doses of the dual EGFR/Her2/neu inhibitor, GW57 (1 μM, 5 μM, or 25 μM). Cell number/lobe was determined by running samples on a Gauva EasyCyte cell counter in the presence of Gauva ViaCount reagent, a cell viability dye. For further details, see Methods and Materials, *Fetal thymic organ culture (FTOC) and analysis*. * indicates $p<0.05$ for two-tailed Student's t-test for GW57-treated lobes compared to DMSO controls. This experiment was performed once.

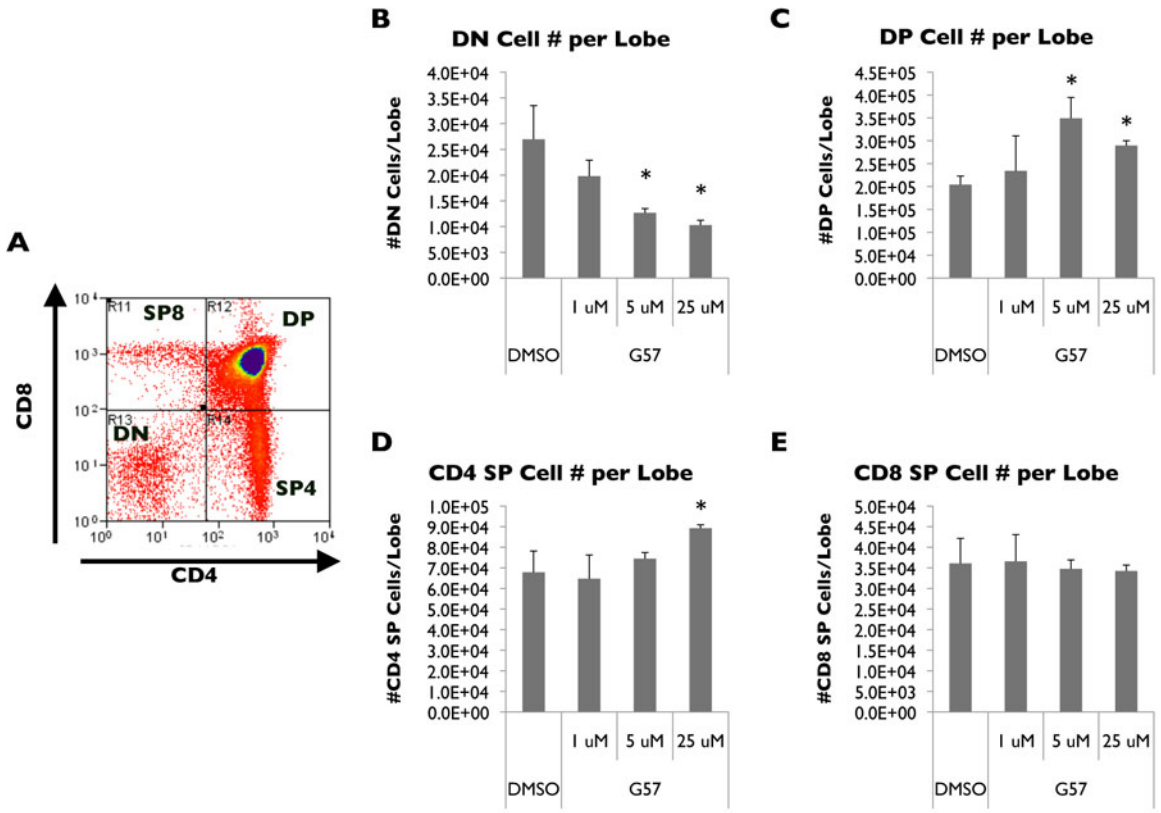


**Figure 3.7: Effect of GW57 on thymocyte differentiation in unmanipulated E14 thymic lobes.**

E14 thymic lobes were cultured and harvested as described in Figure 3.6. Each sample was stained for markers of thymocyte maturation and analyzed by FACs. CD4-FITC, and CD8-PERCP were used to differentiate thymocyte maturation stages. Single stain controls were used to set gate boundaries. The percentage of cells in each quadrant and the total cell number/lobe were used to calculate the number of each cell population per lobe. * indicates $p<0.05$ for two-tailed Student's t-test for GW57-treated lobes compared to lobes in DMSO. This experiment was performed once.

A. Representative CD4 and CD8 staining and boundaries.
B. Effect of GW57 on the number of DN (double negative) thymocytes/lobe.
C. Effect of GW57 on the number of DP (double positive) thymocytes/lobe.
D. Effect of GW57 on the number of CD4 SP (single positive) thymocytes/lobe.
E. Effect of GW57 on the number of CD8 SP thymocytes/lobe.

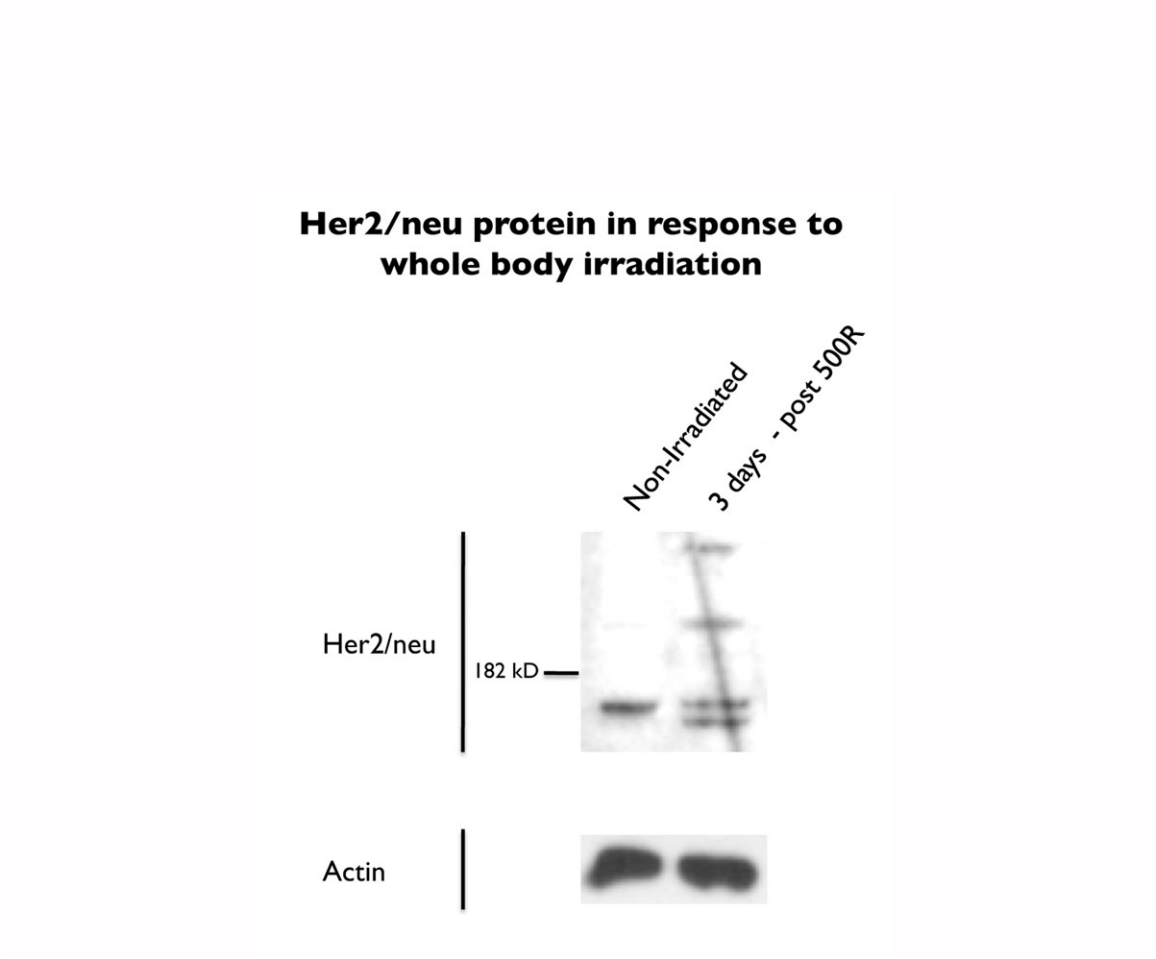


**Figure 3.8: Whole body irradiation increases Her2/neu expression in the thymus.** Adult mice were subjected to 500 Rads γ-irradiation. Three days later, the animals were sacrificed. The thymus organ was removed and stripped of fatty tissue. Thymocytes were removed from the stromal tissue by compressing and twisting the tissue between frosted glass slides. Protein lysates from the remaining thymic stoma were released with NP-40 lysis buffer. Western blot analysis was performed as described in Figure 3.2.

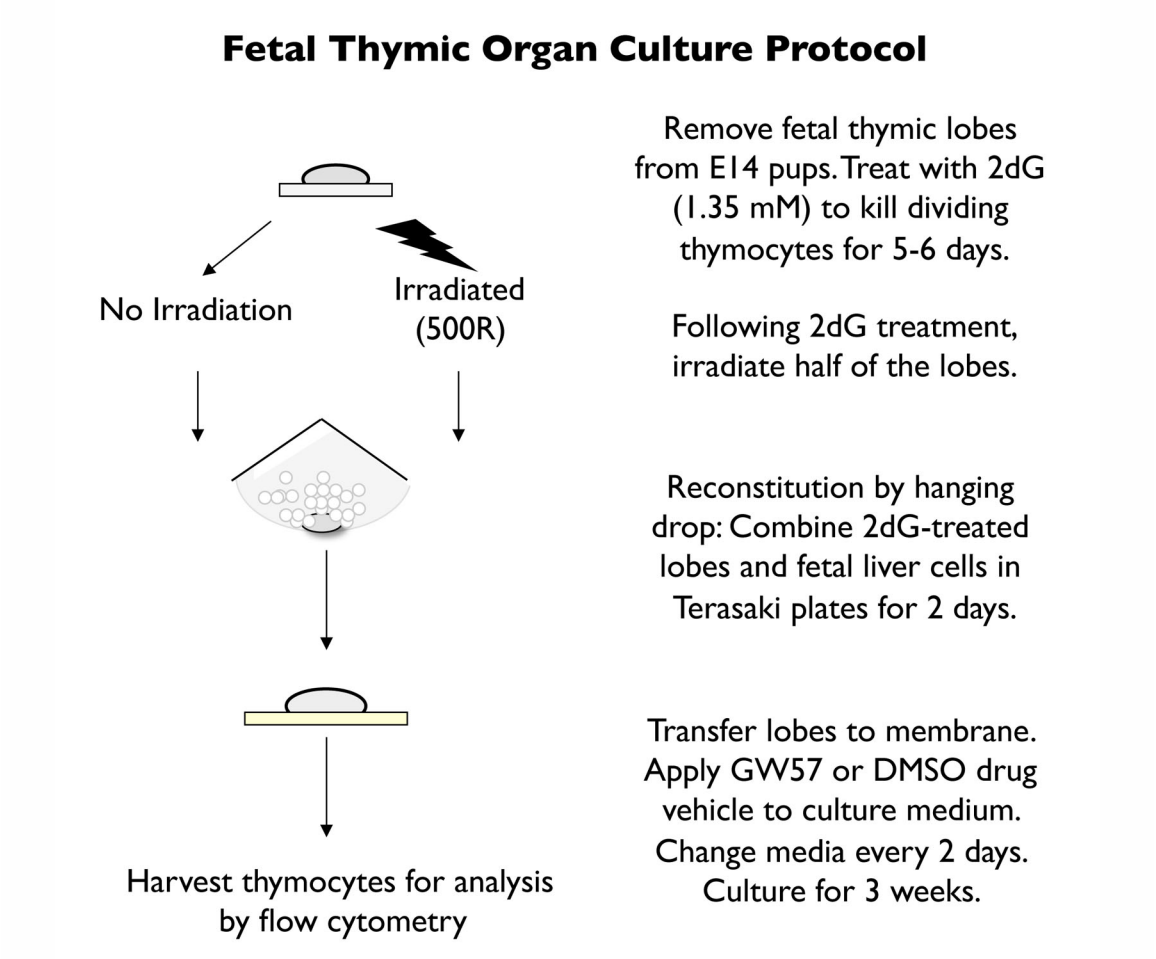


**Figure 3.9: Fetal thymic organ culture (FTOC) experimental design.**
2dG, 2-deoxyguanosine, GW57, GW572016.

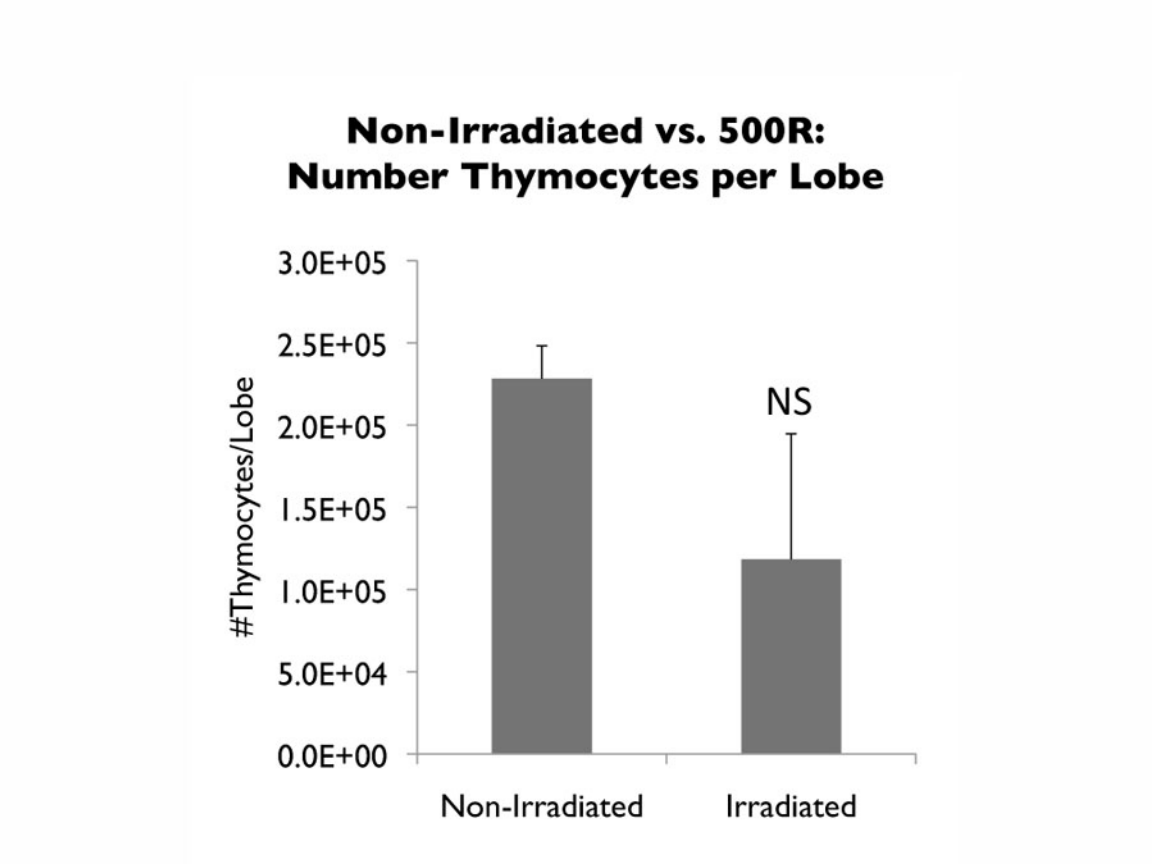


**Figure 3.10: Effects of thymic stroma irradiation on thymocyte output.**
Thymic lobes were treated as outlined in Figure 3.9 and harvested according to the same protocol described in Figure 3.6. The data was subjected to a Student's two tailed t-test using a $p<0.05$ and was not found to be statistically significant (NS, no significance). This experiment was performed once.

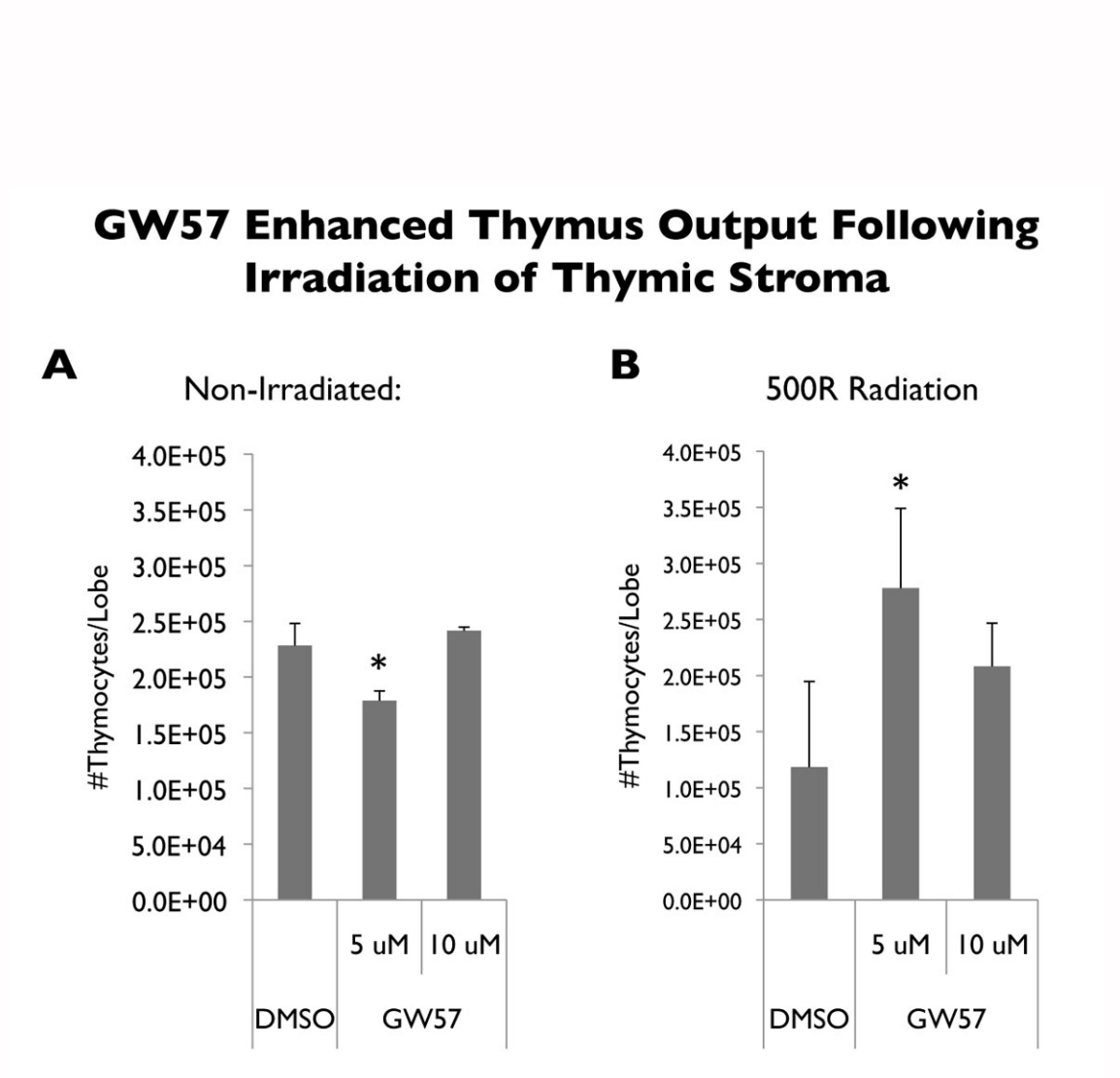


**Figure 3.11: Differential Effect of GW57 on non-irradiated and irradiated lobes.**
Thymic lobes were treated as described in Figure 3.9 and analyzed as described in Figure 3.6. * indicates $p<0.05$ for two-tailed Student's t-test for GW57-treated lobes compared to DMSO controls. This experiment was performed once.

A. Thymocyte number per lobe in non-irradiated lobes.
B. Thymocyte number per lobe in lobes treated with 500R γ radiation.

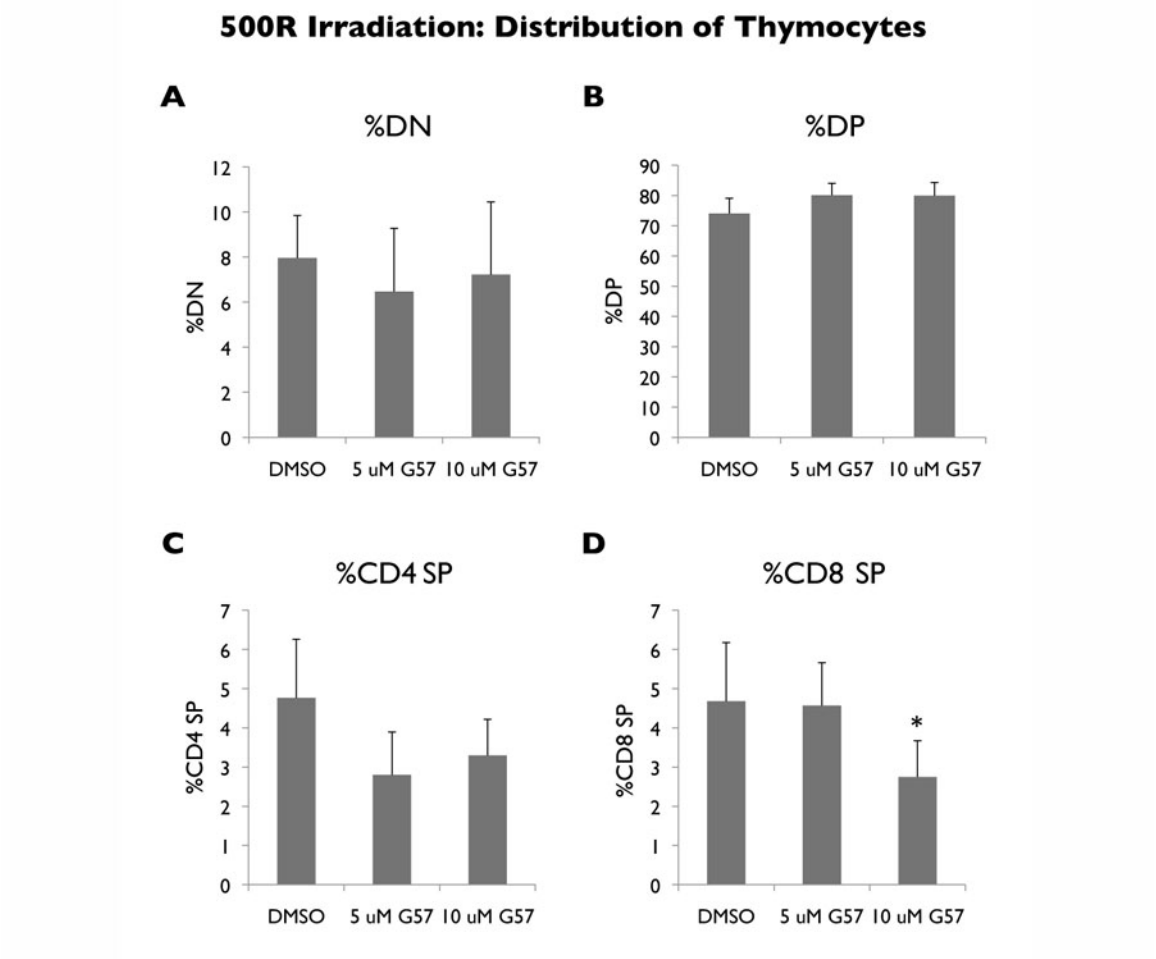


**Figure 3.12: Effect of GW57 on thymocyte differentiation in irradiated lobes.**
Thymic lobes were treated as described in Figure 3.9 and analyzed as described in Figures 3.6 and 3.7. * indicates $p<0.05$ for two-tailed Student's t-test for GW57-treated lobes compared to DMSO controls. This experiment was performed once.

A. Percent double negative (DN) cells.
B. Percent double positive (DP) cells.
C. Percent CD4 single positive (SP) cells.
D. Percent CD8 single positive (SP) cells.

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# CHAPTER FOUR

## FUTURE DIRECTIONS, LESSONS LEARNED, & PERSPECTIVES

## FUTURE DIRECTIONS

### *FOXP3* AND *SIVA* FUNCTION IN T CELLS

In chapter two, I explored the physical and functional interaction between *FOXP3* and *Siva.* The impact of this study would be greatly improved by more primary cell data pertaining to both the biochemical and putative functional interaction.

*Siva and FOXP3 gene regulation in primary CD4 T cells*

I would like to use primary CD4 T cells to evaluate the contribution of *Siva* to gene regulation in T cells and $T_{regs}$. I will test the effects of *Siva* knockdown (KD) on gene expression in sorted $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ T cells. In addition to investigating *IL-2* repression in primary T cells, I would like to look at whether *Siva* KD affects *GITR, CD25,* and *CTLA4,* known *FOXP3* targets associated with $T_{reg.}$function[1,2].

*Refining FOXP3's Siva-interacting domain*

I stated in chapter two that FOXP3 L.Zip point mutants should be tested for Siva binding ability. Additionally, I would like to compare the relative Siva-binding efficiency of *FOXP3-ZnF-L.Zip-Fkh* (AAs 199-431) and *FOXP3-L.Zip-Fkh* (242-431) mutants to the mutants shown in Figure 2.4 (Siva-binding capacity is contained within a central portion of the FOXP3 protein (AAs 106-332)). Also, I would like to make an additional N-terminal truncation (AAs 150-431) because I am interested in the repressor region between FOXP3 AAs 106-199. This transcriptional repressor region is associated with binding between FOXP3 and other gene-repressor partners (HDAC7, TIP60[3], Eos[4], and

NFAT[2]). The Foxp3:p65 interaction, which could be functionally related to the FOXP3:Siva interaction, has not been mapped to a particular FOXP3 domain[5].

*Explanation of Siva mapping data*

The earliest, reproducible Siva:FOXP3 co-immunoprecipitation (Co-IP) datasets showed that the Siva ΔZnF mutant and C-terminus mutants bound to FOXP3. Based on this data, I predicted that the minimal region shared in common by both mutants, the B box domain, would be sufficient to bind FOXP3. Binding between FOXP3 and the Siva B box domain alone is extremely weak, but enhanced compared to the FOXP3 negative control co-IP. The weak binding between FOXP3 and the Siva B box cannot be due to reduced expression levels, as I repeatedly observed high levels of EGFP/Siva B box fusion protein in transfected 293T cell lysates. Another possible caveat is that the Siva B box sequence is short (34 amino acids) and might not form the three dimensional structure that could be necessary for FOXP3-binding when expressed as an EGFP-fusion protein. Binding between the Siva ΔB box mutant and FOXP3 indicates that the B box portion of the C-terminus is not required for Siva binding to FOXP3. The fact that mutants missing either the Zinc-Finger or B box domain sustain FOXP3 binding suggests some redundancy in this region.

## TECHNICAL LESSONS LEARNED

*Siva protein detection issues*

Siva protein detection difficulties limited progress with *FOXP3:Siva* hemi-endogenous co-IP experiments. When I first started the *Siva* project, I performed a series of optimization experiments and determined that the antibody worked best at 1:1,000 in

TBS-0.1% Tween/5% Milk. Even though the antibody worked most of the time, it seems unreliable compared to other antibodies I have used. On the other hand, my impression of antibody unreliability may actually be related to endogenous Siva instability in Jurkats.

When probing Jurkat lysate western blots (WBs) with α-Siva, I routinely observed heavy bands around 50-70 kD and a single band just below the 31 kD marker. Siva is predicted to be 18.8 kD, so I accepted that the 31 kD band was monomeric Siva. Support for this interpretation came from Jurkat cells expressing *shSiva,* where the 31kD band was reduced in the *Siva* KD cells compared to controls. However, I also observed that the 50-70 kD signal intensity decreased slightly in the *Siva* KD cells suggesting that those bands might derive from un-reduced Siva containing complexes, instead of cross-reactive bands. I was not able to resolve this issue because since January, I have not been able to detect either the 50-70 kD bands or the band below 31 kD with the α-Siva antibody.

After returning from vacation last winter, cell recovery from freezing was slow. I noticed excessive amounts of debris in the culture even one week after thawing the cells. After discussing cell viability issues with another colleague who was working with Jurkats, I switched from Fetalplex serum (FPX), a formulated serum complex, to fetal bovine serum (FBS). The Jurkat cell viability immediately improved and I performed transductions in order to express Myc/FOXP3 and carryout the hemi-endogenous co-IP experiments.

At this point, I was testing whether a sepharose bead pre-clear step would minimize the presence of Siva in negative control co-IPs. I obtained weak WB signals for these experiments and I initially thought the problem could be due to a WB reagent or the

pre-clear step. I performed two co-IP experiments before realizing that the Jurkat cells might be the source of my problem.

My concerns about Siva protein stability in Jurkats are based on reports showing that two separate pathways inhibit Siva by ubiquitin-mediated proteasomal degradation. One group showed that LPA growth factor binding to the $LPA_2$ receptor caused Siva degradation in transfected NIH3T3 cells. Siva expression was restored by serum withdrawal[6]. Since, Jurkat cells express the $LPA_2$ receptor[7] and LPA is contained in some serum sources, I suspected that the serum switch might have caused Siva protein degradation. I switched half of the cells I was growing back to FPX for a week and observed a decline in viability. However, when I compared Siva expression between FPX-Jurkat lysates to FBS-Jurkat lysates, I still did not detect the ~31 kD band by WB. The higher MW bands seemed to be slightly enhanced by FPX compared to FBS, but the overall WB intensity was still weaker than what I observed in WBs last fall. This issue remains unresolved.

*Thymopoiesis project*

After moving to a new project, I finally learned where I had previously made mistakes and bad decisions that prevented progress on the thymopoiesis project. The example listed below illustrates how I had a tendency to try short cuts that ultimately led to greater confusion and time wasted.

In addition to the FTOC model described in Chapter Three, I also used the OP9DL1 co-culture system to study thymopoiesis. Based on data discussed in Chapter Three, I had hypothesized that *Foxp3* expression in OP9DL1 cells would affect *in vitro*

thymopoiesis. The experimental design was straightforward: transduce OP9DL1 cells with *mFoxp3* retrovirus or vector and then test OP9DLI-*mFoxp3* cells for their ability to support thymopoiesis. Because OP9DL1 cells already express GFP, I used a retroviral vector, pHSP4, which expresses truncated human CD4 (the intracellular domain was removed leaving only the transmembrane and extracellular domains) as a selection marker.

One of my biggest problems at the beginning of my training was obtaining high titer retrovirus by calcium phosphate transfection. I tried 'fixing' these problems, but ended up creating other obstacles for myself.

When I first transduced OPDL1 cells, the transduction efficiency was very low. A number of protocol changes could have remedied this: titering the retrovirus, improving the retrovirus yield by using lower passage 293T cells, and concentrating retrovirus particles by ultracentrifugation. Multiple factors contribute to successful retrovirus production. At various times, I and other lab members tested calcium phosphate transfection protocol variables (buffers and plasmids), but made limited progress. Eventually, with the help of a new post-doc, we saw that early passage 293T cells would dramatically improve retrovirus production (passage 1-10 vs. passage 30-40), whereas other factors (buffers and plasmids) seem to withstand longer four-degree storage periods without altering retrovirus titer.

Next, I tried to accommodate low transduction efficiency by selecting for transduced clones by limiting dilution (0.5 cells/well in 96-well plates). In retrospect, I do not think this is a good strategy for OP9 cells. According to T. Nakano and colleagues

who have worked extensively with OP9 cells to study embryonic stem cell differentiation, "OP9 cells can easily lose the ability to maintain lympho-hematopoiesis."[8] The OP9DL1 cells may have lost the ability to support hematopoiesis during the clonal selection process. Unfortunately, I did not consider this until afterwards and did not include a non-clonal OP9DL1 monolayer, which would have been the appropriate control. I obtained OP9DL-P4 and OP9DLI-P4-mFoxp3 clones and tested four of them for their ability to support thymocyte differentiation. Two of the Foxp3$^{pos}$ clones failed to form a monolayer by 24-hours after plating. Neither of the remaining clones supported thymocyte differentiation.

## PERSPECTIVE

I spent about three years studying thymopoiesis and another three years investigating the biochemical and functional interaction between *Foxp3* and *Siva.* At the end, I am most curious about *Siva* functions that may be *Foxp3*-independent. Does *Siva* affect thymopoiesis or hematopoiesis? Could *Siva* somehow be implicated in hyper immune activation associated with CD70:CD27 signaling? Answers to some of these questions could inform future investigations regarding a putative functional interaction between *Foxp3* and *Siva*.

### SIVA AND THYMOPOIESIS

The question of *Siva's* potential role in thymopoiesis was first raised by the observation that double positive (DP) thymocytes express high levels of Siva protein[9]. Since then, no reports about Siva function in the thymus have been published.

*Siva* is pro-apoptotic and thymopoiesis is a highly selective process that relies on apoptosis to remove around 97% of thymocytes initiated. Other pro-apoptotic factors are known to sensitize thymocytes to death by neglect.

*Siva's* pro-apoptotic function is associated with Bcl-2 family members and these factors are associated with various thymopoiesis checkpoints. *Siva* promotes mitochondrial-dependent apoptosis by binding and inhibiting Bcl-2 and Bcl-$X_L$. Bcl-2 overexpression rescues thymopoiesis in IL-7 signaling-deficient mice, but neither Bcl-2 nor Bcl-$X_L$ is required for thymopoiesis. Instead, the pro-survival Bcl-2 family member, Mcl-1 provides an essential survival signal during thymopoiesis. *Mcl-1* deficient mice arrest at the DN stage and Siva is highly expressed at the subsequent DP stage, provoking the speculation that the balance between Siva and Mcl-1 contributes to TCRβ selection.

The region of Bcl-2/Bcl-$X_L$ required to interact with Siva has not been identified, so domain information cannot be used to predict whether Mcl-1 and Siva might physically interact. Anti-apoptotic Bcl-2 and Bcl-$X_L$ each contain BH (Bcl-2 homology) domains 1-4 and a transmembrane domain (TMD). Pro-apoptotic Bax contains a TMD and BH domains 1-3. Bax does not interact with Siva, which invites the hypothesis that the BH4 domain could mediate Siva-binding. Mcl-1 is the only known pro-survival Bcl-2 family member that lacks a transmembrane domain; it contains BH1 and BH3 domains[9-13].

Based on the known pro-apoptotic function of *Siva*, I would predict that thymocyte-specific knockdown should positively affect thymocyte number and negatively affect DP thymocyte deletion. This question could be tested in the FTOC

system described in Chapter Three. Given the complex relationship between different thymic lymphoid and stromal cell populations, I would investigate *Siva's* effect on thymocyte distribution by analyzing all known thymic lymphoid populations (double negative, double positive, CD4 single positive, CD8 single positive, $T_{reg}$, natural killer T cells, and γδ T cells). Also, I would perform histology to evaluate thymic cortico-medullary organization.

In addition to possibly contributing to thymopoiesis, gene array data suggests that *Siva* could be involved in hematopoiesis separate from thymopoiesis. The mouse BioGPS gene atlas showed *Siva* expression to be extremely high in stem cell lines and in hematopoietic progenitor cells compared to other cell populations assayed[14,15]. Myelopoiesis can be induced *in vitro* by adding cytokines and growth factors to the culture media. NK, B, and T cell development requires cytokines and stromal cell support. OP9 stromal cells support NK and B cell development because M-CSF is absent[16]. Notch ligand, expression (DL1 or DL4) enables OP9 cells to support T cell development, though not past the DP stage[17]. Therefore, well-characterized experimental systems are available that could be used to study *Siva's* contribution to the differentiation of multiple HSC lineages.

## CD27, SIVA EXPRESSION AND T CELL APOPTOSIS

Siva was first identified as a binding partner for the CD27 co-receptor, but a functional interaction between CD27 and Siva has not yet been observed in T cells under physiological conditions. Siva overexpression in HPB-ALL T lymphocytes enhanced apoptosis in response to CD27 ligation by CD70 *in vitro*[18]. Transgenic mouse data and

adoptive transfer experiments associate the CD27:CD70 axis with chronic T cell activation[19]. *In vitro, Siva* negatively regulates T cells by inhibiting NFκB and IL-2, and by enhancing apoptosis[20,21]. Thus, *in vitro* data predicts that, in the presence of *Siva*, ligation of CD27 by CD70 would promote apoptosis and inhibit T cell activation. Instead, *in vivo* blockade of CD70 protects mice from colitis and arthritis.

One explanation that could resolve Siva *in vitro* data with CD27:CD70 *in vivo* data is inhibition of Siva by ubiquitin-mediated proteasomal degradation. $LPA_2$ (Edg4), a G-protein coupled growth factor receptor and XIAP, an anti-apoptotic E3 ligase, both inhibited Siva function by ubiquitination and proteasome-dependent mechanisms *in vitro*[6,20]. $LPA_2$-mediated Siva degradation required LPA stimulation. Growth factor withdrawal restored Siva expression[6]. Though the $LPA_2$ experiments were not performed in a lymphocyte line, $LPA_2$ is expressed by T cells and has been shown to mediate T cell migration[7]. A possible physiological scenario could exist where LPA supports survival of $CD27^{pos}$ T cells by inhibiting Siva expression. Support for such a possibility requires more data pertaining to the expression kinetics and immunobiology of LPA, $LPA_2$, Siva, CD27, and CD70.

## FINAL COMMENT

For a long time I was attracted to big questions that seemed to necessitate complex model systems and experimental time courses requiring weeks or months. But once I started to generate reliable data for myself using simple transcriptional assays, my attraction to *in vivo* systems diminished. I finally experienced the thrill that can happen when there is a steady pace between the idea, the hypothesis, an experiment, the data, and

back again. I expect that if I only did three-day long luciferase assays, I would soon get bored, but it is tempting to exhaust any experimental system that yields quick and reliable results. Even so, the limitations of *in vitro* systems need to be respected and models inspired by *in vitro* data should be subjected to questions concerning *in vivo* relevance.

I've always been comfortable and relatively succussful in traditional academic settings. Bench science was challenging for me, but I do not think I could have learned the lessons I gained in any other way. One must learn to balance the rational with the irrational. That is, one must do one's best to logically incorporate controls to accommodate all known variables, but be able to accept that unaccounted variables may remain. That is where discovery happens. If we insist on being able to explain why every result is the way it is, then we may blind ourselves to learning something that lies outside of our predictions.

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