The role of Ikaros in Foxo1-driven gene expression in CD4 T cells
THE ROLE OF IKAROS IN FOXO1-DRIVEN GENE EXPRESSION IN CD4 T CELLS

by

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DEDICATION

I would like to dedicate this work to my parents, brother and loving fiancée. Thank you for believing in me and being my greatest support.
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I have several people to acknowledge, who have helped me achieve this milestone. First and foremost, I would like to thank my advisor, Dr. Susan Winandy. She has been such an inspiration and has been extremely supportive. She has taught me to think critically and independently, allowing me to gain a strong understanding of my studies. I am truly grateful for her mentorship.

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THE ROLE OF IKAROS IN FOXO1-DRIVEN GENE EXPRESSION IN CD4 T CELLS

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ABSTRACT

The existence of a robust, mature CD4 T cell population is essential in orchestration of an immune response. CD4 T cell activation is a result of antigenic stimulation of a unique cell pool that is normally resting. Termed “naïve”, these CD4 T cells lack effector function and are maintained long term in the periphery. Expression of key cell surface receptors and transcription factors dictates their ability to survive, home and differentiate into effector subsets. However, transcriptional regulation of these processes in naïve CD4 T cells is only partly characterized.

Ikaros has been identified as a transcriptional activator and repressor of T cell lineage fate decisions and polarization into T helper cell subsets. In this dissertation, a role for Ikaros in regulation of naïve CD4 T cells is revealed as in its absence, cells exhibit decreased survivability, impaired migration to lymph nodes and failure to develop into induced regulatory T cells (iTreg). Defects are linked to decreased expression of IL-7Rα, CD62L and Foxp3, respectively, all identified as targets of a transcription factor important in naïve CD4 T cell homeostasis, Foxo1. Analogous consequences on T cell survival, homing and differentiation have been reported for Foxo1-deficient T cells.
Furthermore, results from Western blot and qRT-PCR analyses of protein and mRNA from Ikaros null (IK-/-) CD4 T cells demonstrated decreased Foxo1 levels, prompting investigation into mechanisms for regulation of Foxo1 expression by Ikaros. Retroviral transductions were performed, beginning with delivery of Ik-7 and Foxo1-shRNA, interfering with Ikaros and Foxo1 activity in wild type cells, respectively. Similar decreases in CD62L and IL-7Rα levels indicated the need for both Ikaros and Foxo1 for expression. However, re-introduction of either Foxo1 or Ikaros into IK-/- CD4 T cells highlighted differential modes of Ikaros and Foxo1 regulation for IL-7Rα and CD62L expression. qRT-PCR analyses revealed increased levels of Foxo1 mRNA with Ikaros transduction into IK-/- CD4 T cells. My studies have thereby identified Ikaros to be the first transcriptional regulator of Foxo1 gene expression in ensuring survival, homing and iTreg differentiation of the naïve CD4 T cell compartment.
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CHAPTER 1. INTRODUCTION

1.1 CD4 T Helper Cells and the Immune Response

An immune response is defined by how our body is able to recognize and mount defenses against foreign antigens. It is important for there to be a strong, robust lymphocyte population to allow orchestration of antigen-specific responses, ultimately resulting in long-lasting immunity. The lymphocyte population consists of B lymphocytes (B cells) and T lymphocytes (T cells). T cells mediate cellular immunity, whereas B cells mediate humoral immunity. Together, both populations provide adaptive immunity, working in close collaboration with cells of the innate immune system.

CD4 T helper cells and CD8 cytotoxic T cells make up the majority of the T lymphocyte population. CD4 T cells are organizers of the immune response. Once presented with antigen by antigen presenting cells (APC), CD4 T cells direct other immune cells to initiate and maintain effective humoral and cellular immunity. They assist in antibody production by B cells, and regulate CD8 T cell responses and macrophage function (Zhu et al. 2010). CD4 T cells are also important mediators of immunological memory(Jenkins et al. 2001). Defects in CD4 T cell development lead to severe immunodeficiency and can be fatal.

Following migration from the thymus, mature CD4 T cells that have not encountered antigen are maintained in a “naïve” state in the periphery. These cells are resting, as they are not activated and as a result do not perform effector functions (Luckheeram et al. 2012). However, it is important to maintain this cell population of
CD4 T cells, as they possess unique antigen receptors and functionality. Naïve T cells survive for long periods of time in circulation, requiring cytokine signals delivered by IL-7 and TCR recognition of self-major histocompatibility complex (MHC) (Schluns et al. 2000; Takeda et al. 1996). These signals trigger processes of homeostatic proliferation as revealed by experiments involving adoptive transfer of naïve T cells into lymphopenic mice. These mice received multiple rounds of radiation resulting in a complete depletion of B and T cell populations (Ernst et. al 1999; Tanchot et. a 1997). Upon transfer, naïve T cells not only survived but also actively proliferated to fill up the niche (Tanchot et al. 1997). T-cell lymphopenia often occurs after chemo- or radiotherapy, or after certain infections, including human immunodeficiency virus (HIV). Therefore, understanding the molecular mechanisms involved in maintenance of naïve CD4 T cells could lead to discovery of new therapeutic interventions.

**CD4 T Cell Development in the Thymus**

T cell precursors originating in the bone marrow leave and travel to the thymus for maturation (Takahama 2006). Once in the thymus, TCR gene rearrangement and thymocyte selection processes prepare mature T cells for antigen recognition. At the earliest stage of development, T-cell precursors have a double negative (DN) (CD4-CD8-) phenotype. At the end of this stage, immature single-positive (ISP) cells (CD4+CD8- or CD4-CD8+) begin to rearrange the the TCRβ gene to form a pre-TCR (Gameiro et al. 2010). Pre-TCR signaling ensures survival and further maturation towards the double positive (DP) (CD4+CD8+) subset of thymocytes (Wang et al. 2011). At this point,
positive and negative selection events occur, determining the selection of a mature T-cell repertoire.

During positive selection, double-positive thymocytes (CD4+ CD8+) interact with MHC molecules present on the cortical epithelium. This event leads to the loss of one co-receptor molecule (CD4 or CD8) (Klein et al. 2009; Starr et al. 2003), generating single-positive (SP) thymocytes (CD4+ or CD8+). Negative selection allows for the elimination of auto-reactive T cells, important in preventing the onset of autoimmunity. Within the thymus also resides a population of Foxp3-expressing CD4+CD25+ regulatory cells known as natural regulatory T cell (nTregs), which are important in the maintenance of self-tolerance. nTregs will be discussed in more detail in following sections.

Mature naïve SP CD4 T cells are then released into the periphery, at which point they are free to migrate to secondary lymphoid organs. Within the spleen, lymph nodes, and the mucosa associated lymphoid tissue (MALT), they search for peripheral MHC class II molecules on the surfaces of APCs (Takahama 2006). TCR engagement occurs through interaction with an antigen-MHC on the surface of dendritic cells (DCs). The principal function of DCs is to present antigens, and provide essential costimulatory signals for T cell activation. Importantly, only DCs have the ability to induce a primary immune response in resting naïve T lymphocytes (Pozzi et al. 2005).

\textit{CD4 T Cell Differentiation in the Periphery}

Once in the periphery, CD4 T cells can differentiate into a variety of effector subsets, that include Th1, Th2, Th17, iTreg and the more recently characterized follicular
helper T (Tfh) cells (Zhu et al. 2010). CD4 T cell differentiation is regulated by an extensive network of cytokines and transcription factors. Cytokines in the microenvironment as well as surrounding APCs trigger expression of a unique transcription factor, or so called master regulator, that in turn, functions to regulate cytokine expression as well as other genes specific to a particular subset (Table 1).

**Th1 Cell Differentiation**

Th1 cells provide immunity against intracellular pathogens. IL-12 and IFN-γ are the key differentiating cytokines that trigger downstream signaling pathways in development of Th1 cells (Trinchieri et al. 2003). Following activation via pattern recognition receptors, DCs secrete IL-12 (Steinman et al. 2003; Iwasaki and Medzhitov 2004). IL-12 signals through the IL-12 receptor complex composed of the IL-12Rβ1 and IL-12Rβ2 chains (Presky et al. 1996). The IL-12R complex is coupled to the Jak-STAT signaling pathway, inducing phosphorylation of transcription factor signal transducer and activator of transcription 4 (STAT4) (Bacon et al. 1995). Mice deficient in IL-12, the IL-12R complex or STAT4 have profoundly diminished Th1 responses *in vitro* and *in vivo* (M H Kaplan et al. 1996).

The Th1 signature cytokine, IFN-γ, binds and initiates downstream activation of STAT1, a pathway important in suppressing the development of Th2 and Th17 cells (Lugo-Villarino et al. 2003; Lazarevic et al. 2011). Mice deficient in IFN-γ or STAT1 demonstrate an increased susceptibility to intracellular pathogens and viruses (Dalton et al. 1993). TCR signaling and the IFN-γ/STAT1 signal transduction pathways induce expression of the transcription factor T-bet, known as the master regulator for Th1
differentiation. T-bet in turn directly induces production of IFN-\(\gamma\) via remodeling of the \textit{ifng} gene (Szabo et al. 2000). T-bet deficient mice do not produce IFN-\(\gamma\) and as a result cannot overcome infection with intracellular bacteria such as \textit{Mycobacterium tuberculosis} and \textit{Leishmania major} (Vahedi et al. 2013; Mark H. Kaplan et al. 1996).

T-bet and IFN-\(\gamma\) are essential in promoting Th1 responses, while inhibiting factors important in differentiation into alternative lineages. Mice with homologous disruption of the \textit{ifng} gene on the C57BL/6 background were infected with \textit{Leishmania major} and the immune response was assessed. The knockout mice developed CD4 T cells that contained transcripts for IL-4, IL-5, and IL-13, effector cytokines produced by Th2 cells (Wang et al. 1994). These data suggest that CD4 T cells that normally respond to antigens by differentiation to Th1 cells default to the Th2 pathway in the absence of IFN-\(\gamma\). In addition, exogenous T-bet introduction into CD4 T cells prevents production of Th2 cytokines, instead encouraging cells to produce IFN-\(\gamma\) (Szabo et al. 2000; Szabo et al. 2002). The Th2-suppressive activity of T-bet has also been confirmed in the absence of IFN-\(\gamma\), indicating that T-bet has inhibitory function independent of IFN-\(\gamma\) stimulation (Oh and Hwang 2014).
A CD4 T cell becomes activated after antigen presentation. Depending on the microenvironment of activation, key differentiation cytokines trigger expression of transcription factors. Master regulators are factors which initiate differentiation of CD4 T cells into specific polarized subsets. Cells belonging to each subset secrete effector cytokines. Effector cytokines then program surrounding cells via downstream mediators. Such transcriptional networks provide protection against various intracellular and extracellular pathogens, while also maintaining self-tolerance.

### Table 1. Key cytokines and transcription factors important in CD4 T cell differentiation and function.

<table>
<thead>
<tr>
<th>CD4 Subset</th>
<th>Differentiation Cytokines</th>
<th>Master Regulators</th>
<th>Effector Cytokines</th>
<th>Downstream mediators</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, IFN-γ</td>
<td>T-bet</td>
<td>IFN-γ, IL-2, TNF-α/β</td>
<td>STAT1, STAT4</td>
<td>Protection against intracellular pathogens</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-2</td>
<td>GATA3</td>
<td>IL-4, IL-5, IL-9, IL-10 and IL-13</td>
<td>STAT6, STAT5</td>
<td>Protection against extracellular parasites</td>
</tr>
<tr>
<td>Th17</td>
<td>TGF-β, IL-6, IL-21, IL-23</td>
<td>RORγt</td>
<td>IL-17A, IL-17F, IL-21, and IL-22</td>
<td>STAT3</td>
<td>Protection against extracellular bacteria and fungi</td>
</tr>
<tr>
<td>Treg</td>
<td>TGF-β, IL-2</td>
<td>Foxp3</td>
<td>IL-10</td>
<td>Smad2, Smad3, Smad4, STAT5</td>
<td>Maintenance of self-tolerance</td>
</tr>
<tr>
<td>Tfh</td>
<td>IL-6, IL-21</td>
<td>Bcl6</td>
<td>IL-21</td>
<td>STAT3</td>
<td>B cell help</td>
</tr>
</tbody>
</table>
**Th2 Cell Differentiation**

Th2 cells provide immunity against extracellular parasites through production of Th2 cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (Vahedi et al. 2013). IL-4 initiates Th2 cell differentiation through phosphorylation and nuclear translocation of the transcription factor STAT6, which activates transcription of the gene encoding the master regulator, GATA-binding protein 3 (GATA-3) (Kaplan et al. 1996; Zhu et al. 2001). GATA-3 then initiates Th2 cytokine production by directly binding to the *Il5*, *Il13* and *Il10* promoter and *Il4* enhancer regions (Ansel et al. 2006). STAT5, activated downstream of IL-2 signaling, has also proven to be essential in Th2 differentiation. Retroviral-mediated expression of a constitutively active *Stat5A* mutant led to restored IL-4 production in the absence of IL-2 and IL-4/STAT6 signaling in IL-4Rα-deficient cells (Zhu et al. 2003). In addition, cells expressing both GATA-3 and STAT5, cultured under Th1 or Th2 conditions, had the highest percentage and mean fluorescence intensity (MFI) of IL-4 producing cells (Zhu et al. 2003). Therefore, combined transcriptional regulation by GATA-3 and STAT5 have proven effective in Th2 lineage commitment. GATA-3 is required for the initiation and maintenance of Th2 responses both *in vitro* and *in vivo*. In GATA-3 deficient mice, differentiation of naive cells is diverted towards the Th1 lineage (Zhu et al. 2004). In addition, GATA-3 suppresses Th1 differentiation by down regulating STAT4 and T-bet expression, which contributes to decreased IFN-γ production (Zhu et al. 2006; Usui et al. 2003). GATA-3 represses IL-12 induced Th1 development, however only during initial stages of naïve T cell programming. As early as 7 days after
Th1 priming, retroviral transfection of GATA-3 into Th1 developing cells fails to induce IL-4 production (Ouyang et al. 1998). In conclusion, GATA-3 expression is essential to maintaining Th2 cells, preventing onset of differentiation into alternative phenotypes.

**Th17 Cell Differentiation**

A third major effector CD4 T cell population, designated Th17 cells, defend against extracellular bacteria and fungi. CD4 T cells differentiate into Th17 cells in the presence of TGF-β and IL-6. TGF-β and IL-6 together induce activation of the Rorc gene via STAT3 activation (Manel et al. 2008; Ivanov et al. 2006; Yang et al. 2008). This gene encodes RORγt, termed the master transcriptional regulator of Th17 cells as it drives the expression of IL-17A, IL-17F, and IL-22 (Ivanov et al. 2006; Yang et al. 2008). These cytokines stimulate the expression of pro-inflammatory mediators and anti-microbial peptides.

The Th17 differentiation process is initiated by TGF-β and IL-6, followed by a self amplification stage dependent on IL-21, ending with the IL-23 regulated stabilization of the Th17 phenotype. Initially, IL-23, a cytokine also produced by DCs, together with IL-6 were thought to be the main drivers of Th17 differentiation as they both activate STAT3 (Yang et al. 2007). However it was later found that IL-23 is not required for naïve T cell differentiation into Th17 cells, but is required for stabilizing the phenotype (Weaver et al. 2007). IL-23 receptor is expressed on CD4 T cells only after they have been stimulated with IL-6 and TGF-β (Bedoya et al. 2013). Also, Th17 cells will still develop in the presence of IL-23-blocking antibodies as long as IL-6 and TGF-β are
present (Bedoya et al. 2013). TGF-β and IL-6 were then established as the critical cytokines for early commitment to the Th17 lineage.

Unlike IFN-γ for Th1 cells or IL-4 for Th2 cells, IL-17 produced by Th17 cells does not amplify differentiation. Instead IL-21, another cytokine highly expressed by Th17 cells, induces IL-17 production and expression of RORγt. In return, IL-21 is known to activate STAT3 and its ability to induce Th17 differentiation is abrogated in the absence of STAT3 (Wei et al. 2007). This highlights autocrine signaling properties of IL-21 in encouraging Th17 lineage commitment. IL-21 also works to prevent Th1 development by suppressing Ifng and Tbx21 (Suto et al. 2006). IL-21 together with IL-6, also inhibits iTreg development by suppressing Foxp3 expression, while greatly enhancing IL-17 production (Nurieva et al. 2007).

**Regulatory T Cell Differentiation**

In the mid to late 1990s, numerous studies identified CD4+ CD25+ regulatory T cells (Tregs) as a novel subset of “regulatory” cells, possessing the ability to suppress adaptive T cell responses and prevent autoimmunity (Sakaguchi et al. 2007; Bloom et al. 1992; Groux et al. 1997; Chen et al. 1994). Tregs were later found to express the signature Forkhead transcription factor 3 (Foxp3), important for development and regulatory function (Hori et al. 2003; Fontenot et al. 2003). Mutations in Foxp3 in humans causes immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) resulting in severe autoimmune diseases, inflammatory bowel disease, and allergy (Bennett et al. 2001). Likewise, mice carrying a mutation of Foxp3 (scurfy)
are deficient in Tregs and develop systemic autoimmunity (Fontenot et al. 2003; Bennett et al. 2001). These fatal outcomes highlight an important role for Foxp3 in the development of functional Tregs.

Foxp3 expressing Treg subsets include natural Tregs (nTregs) and induced Tregs (iTregs) (S Sakaguchi et al. 1995). nTregs originate from the thymus as CD4 T cells expressing high levels of CD25 together with Foxp3. nTregs are essential for maintaining immune tolerance and homeostasis (Sakaguchi et al. 1995). iTregs originate from the thymus as single-positive CD4 cells. They differentiate into CD25 and Foxp3 expressing Tregs following antigenic stimulation. Both regulatory subsets produce immunoregulatory cytokines such as TGF-β and IL-10 (Chen et al. 2003). iTregs suppress immune responses at sites of inflammation, especially at the mucosal surfaces.

TGF-β and IL-2 are the cytokines important for iTreg cell lineage commitment (Josefowicz and Rudensky 2009). Foxp3 is induced downstream of TGF-β signaling. Ligand binding to TGF-βR initiates phosphorylation events leading to formation of a complex composed of TGF-βR1 and TGF-βR2, both transmembrane receptor serine/threonine kinases. The phosphorylated kinases proceed to activate Smad2 and Smad3, which complex with Smad4, and together bind to sites in an enhancer region of the Foxp3 gene (Martinez et al. 2009; Feng and Derynck 2005). Smad3-deficient T cells have a profound defect in Foxp3 expression upon iTreg induction (Moore et al. 2001). Smad3 directly binds RORγt, inhibiting its transcriptional activity (Martinez et al. 2009). Therefore, Smad3 has been shown to differentially enhance iTreg development by inducing Foxp3 expression and inhibiting Th17 differentiation.
IL-2R signaling enhances iTreg development. STAT5 is activated downstream of IL-2R. It directly binds the Foxp3 promoter and enhances Foxp3 expression (Burchill et al. 2007). In addition, activated STAT5 antagonizes TGF-β and IL-6-mediated STAT3 binding to the il17 locus sites, resulting in enhanced iTreg differentiation (Moore et al. 2001; Kühn et al. 1993). Therefore, differentiation of iTregs requires both IL-2 and TGF-β signaling pathways to be active.

Tregs produce IL-10, an anti-inflammatory cytokine involved in immunosuppression of the effector function of T cells, monocytes, macrophages and DCs. IL-10 induces downregulation of MHC class II, and the co-stimulatory molecules CD80 and CD86 on APCs. In addition, IL-10 represses production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNFα (Moore et al. 2001). The importance of IL-10 in limiting inflammation is evident in IL-10/-/- mice as they suffer from inflammatory diseases that include inflammatory bowel disease (Moore et al. 2001; Kühn et al. 1993).

**Tfh Differentiation**

Follicular T helper cells (Tfh) are a more recently described subset of CD4 T cells. Located in follicular areas of lymphoid tissue, they contribute to antigen-specific B-cell immunity (Vinuesa et al. 2005; Breitfeld et al. 2000). Tfh cells were originally identified through their high expression of CXCR5, a chemokine receptor normally found on B cells, which allows these cells to migrate to the B cell follicle (Breitfeld et al. 2000).
IL-6 and IL-21 are the main cytokines involved in Tfh differentiation. IL-6R signaling induces Bcl6 expression by newly activated CD4 T cells (Nurieva et al. 2009). Bcl-6 is the master transcription factor that controls Tfh cell differentiation (Yu et al. 2009). IL-21 is also secreted by Tfh cells and is important for allowing Tfh cells to provide B cell help. IL-21–induced STAT3 activation down-regulates Bcl6 and up-regulates expression of Blimp1 (Diehl et al. 2008). Blimp1 specifically inhibits Bcl6, thereby promoting plasma cell differentiation (Johnston et al. 2009). Bcl-6 also acts as a transcriptional repressor of Th1, Th2 and Th17 cell lineages (Yu et al. 2009). Chromatin immunoprecipitation (ChIP) assays on human Tfh cells revealed high ChIP enrichment ratios values for Bcl6 across the Tbx21 (encoding T-bet) and Rorc (encoding RORγt) promoters (Yu et al. 2009).

Expression of other differentiation programs can occur within germinal center (GC) Tfh cells. For example, during a strong Th1-mediated response, as elicited by lymphocytic choriomeningitis virus (LCMV), Tfh cells express IFN-γ (Yusuf et al. 2010). In autoimmunity prone mice (BDX2), spontaneous GC development is associated with IL-17-producing Tfh cells (Ding et al. 2013). Therefore, Tfh cells display alternative phenotypes depending on environmental cues, causing speculation of whether or not they are their own lineage at all.

### 1.2 Ikaros and CD4 T Cell Development

After CD4 T cell activation, developmental programs are switched on allowing differentiation into corresponding T helper cell subsets. Transcription factors
dictate each of these programs. Ikaros is one such factor, acting as both a transcriptional activator and repressor in regulation of multiple programs of CD4 T cell differentiation and function. These programs include Th cell lineage fate decisions and regulation of proliferative responses.

Ikaros family members are expressed in lymphoid-like cells of the most primitive of vertebrate immune systems, such as hagfish and lampreys, but have not been found in *Drosophila melanogaster* or *Caenorhabditis elegans* genomes (Rothenberg and Pant 2004). However, Ikaros does contain C2H2 zinc finger protein arrangements similar to *Hunchback*, a Drosophila segmentation gap protein (Large and Mathies 2010). Ikaros was first identified in 1992 in studies aimed at defining early events in T cell differentiation, which involved examining transcriptional control of the CD3δ gene (Georgopoulos et al. 1992). Ikaros was discovered using an expression library screen for proteins that bound an enhancer for the CD3δ gene (Georgopoulos et al. 1992). Concurrently, the Ikaros gene *Ikzf1* was found to encode a protein that interacted with the promoter of the lymphocyte specific *terminal deoxynucleotidyltransferase (TdT)* gene, Lyf-1, highlighting its lymphoid-specific role (Lo et al. 1991). Studies since then have been involved in investigating the role of Ikaros regulation during multiple stages of hematopoietic development.

*The Ikaros Gene*

The Ikaros gene (*Ikzf1*) encodes seven translated exons, which undergo alternative splicing events generating isoforms that vary in function and expression (Molnár et al.
Ikaros is a zinc finger DNA-binding protein consisting of two clusters of Cys$_2$-His$_2$ zinc fingers. Each isoform differs in the number of zinc fingers in the amino (N)-terminal DNA-binding domain. The N-terminal zinc fingers allow for sequence specific DNA binding to the Ikaros consensus sequence G-G-G-A-A, and at least 3 of the N-terminal zinc fingers are required for DNA binding (Molnár and Georgopoulos 1994). Within exon 7 of each isoform, the two C-terminal zinc fingers make up the dimerization domain allowing for protein-protein interactions. The formation of homo- and heterodimers among the DNA binding isoforms (i.e. Ik-1, Ik-2, Ik-3) increases their affinity for DNA whereas formation of heterodimers with isoforms without an intact DNA binding domain (i.e. Ik-4, Ik-5, Ik-6, Ik-7) do not bind DNA and are transcriptionally inactive (Sun et al. 1996; Georgopoulos et al. 1992). Therefore, Ikaros proteins with fewer than three N-terminal zinc fingers can exert a dominant-negative effect by interfering with the activity of Ikaros isoforms that can bind DNA (Sun et al. 1996).

The Ikaros gene is expressed in all hematopoietic cells and is differentially expressed in the thymus and spleen. Ikaros is more highly expressed in thymocytes in comparison to splenocytes, suggesting that Ikaros may function to regulate gene expression in T cells and their progenitors (Georgopoulos et al. 1992). Importantly, Ikaros is 95% conserved in humans and mice at the protein level (Molnár et al. 1996). There are four other members of the Ikaros family of zinc finger DNA binding factors. Helios and Aiolos, like Ikaros, are expressed primarily in hematopoietic cells, while the
other two members, Eos and Pegasus, are expressed more variably in tissues (Hahm et al. 1998; Kelley et al. 1998; Morgan et al. 1997; Perdomo et al. 2000; Honma et al. 1999).
Figure 1. Alternative splicing generates Ikaros isoforms with varying DNA binding affinities and function.

Within the DNA binding domain, each isoform differs in the number of N-terminal zinc fingers. This results in unique DNA binding capabilities of each isoform. However, the number of zinc fingers in the C-terminal dimerization domain are constant, allowing each isoform to partake in formation of homo- and heterodimers. Isoforms with less than three N-terminal zinc fingers (Ik-4 to 8) cannot bind DNA, and dimerization with with DNA-binding isoforms (Ik-1 to 3) results in a dominant negative role in transcription (Sun et al.1996; Georgopoulos et al. 1992). Ik-7 exerts a dominant-negative effect, and is not a naturally occurring isoform.
**Ikaros-deficient mouse models**

The generation of multiple mouse models with Ikaros deficiency has allowed for identifying the role of Ikaros in critical developmental processes (Figure 2). The initial mouse model used to study Ikaros was the dominant negative (Ik\(^{DN/DN}\)) model. Exons 3 and 4 of the Ikaros gene \textit{ikzf1} were deleted, removing three of the four N-terminal zinc fingers of the DNA binding domain (Winandy et al. 1995; Georgopoulos et al. 1994). This deletion therefore results in the production of a non-naturally occurring isoform Ik-7, which exerts a dominant negative effect on functional isoforms. Mice homozygous for the mutation die early within 1-3 weeks of age. Developmentally these mice lack B cells, T cells, natural killer cells and DCs (Georgopoulos et al. 1994). 100% of heterozygous mice develop T cell leukemias by twelve weeks of age (Winandy et al. 1995).

The second Ikaros mouse model generated was the Ikaros null (IK-/-) mutation in which exon 7 was deleted (Wang et al. 1996). This deletion results in a complete lack of Ikaros protein. IK-/− mice lack B cells, natural killer cells and fetal T cells, and have decreased numbers of DCs, \(\alpha\beta\) and \(\gamma\delta\) T cells (Georgopoulos et al. 1994). These mice also lack lymph nodes. Although T cells develop in IK-/− mice, there is an abnormally high ratio of CD4+ to CD8+ T cells (Urban and Winandy 2004). Mature T cells display lower thresholds of activation in response to TCR stimulation, thereby requiring less antigenic stimulation to proliferate (Georgopoulos et al. 1994). Studies have reported that this lower threshold of TCR signaling in IK-/− mice leads to enhanced positive selection towards the CD4 lineage (Georgopolous et al. 1997;
Urban and Winandy 2004).
Figure 2. Ikaros-deficient mouse models.

Mouse models have been generated to study the function of Ikaros. Deletion of exons 3 and 4 led to formation of the dominant negative (Ik$^{\text{DN/DN}}$) model. These mice exhibit severe developmental defects that include a complete lack of B cells, T cells, NK cells and DCs. This deletion results in the production of a non-naturally occurring isoform identical to Ik-7, which is known for its dominant negative effect on functional isoforms. The second Ikaros mouse model involved deletion of exon 7, resulting in an Ikaros null (IK$^{-/-}$) mutation. This deletion results in a complete lack of Ikaros protein. Similar developmental defects are observed as the Ik$^{\text{DN/DN}}$ model, however not as severe. IK$^{-/-}$ mice lack B cells, natural killer cells and fetal T cells, and decreased DCs and and $\alpha\beta$ and $\gamma\delta$ T cells. These mice also lack lymph nodes. IK$^{-/-}$ thymocytes also display defects in negative selection (Urban and Winandy 2004). Lastly, clonal T cell populations expand in the thymus at five to six weeks of age.
Transcriptional regulation by Ikaros during T cell development

Ikaros functions as both a transcriptional activator and repressor through its interaction with components of chromatin remodeling complexes, including the Nucleosome Remodeling and Deacetylase (NuRD) and SWI/SNF complexes (Kim et al. 1999). These are large multi-protein complexes, that together with histone deacetylases (HDAC) or histone acetyltransferases (HAT) can influence transcription, by altering the accessibility of DNA. The Nucleosome Remodeling and Deacetylase (NuRD) complex associates with HDACs, creating an inaccessible chromatin conformation. In contrast, the SWI/SNF complexes, in collaboration with HATs, expose DNA to transcription factors, thus permitting transcription.

A specific role for Ikaros in regulation of gene expression in T cell development has been defined. Chromatin immunoprecipitation (ChIP) experiments have shown that Ikaros can bind regulatory regions in the CD8α gene (Harker et al. 2002). Decreased Ikaros levels result in fewer CD8 SP and DP cells in mice, suggesting that Ikaros positively regulates CD8 expression. A role for Ikaros in CD4 expression has also been described. In thymocytes, Ikaros binds to the silencer regions of the CD4 locus (Naito et al. 2007). Ikaros null thymocytes at the late double negative stage express CD4, suggesting that Ikaros represses CD4 expression at these stages.

The ability of Ikaros to acts as a transcriptional activator and repressor has also proven essential in regulation of CD4 T cell differentiation processes in the periphery. Under Th2-promoting culture conditions, Ikaros activity is required to silence ifnγ gene expression in CD4 T cells (Quirion et al. 2009). Ikaros achieves this in part by binding to
and repressing transcription of the *tbx21* gene, which encodes the Th1 master regulator, T-bet (Quirion et al. 2009). Therefore, IK-/- CD4 T cells are unable to express normal amounts of IL-4, IL-5 and IL-13 and instead acquire an IFN-γ-producing Th1-like phenotype (Quirion et al. 2009). These cells show histone 3 (H3) hypoacetylation across the Th2 locus (Lee et al. 2002), as well as decreased expression of GATA-3 and an increase in expression of T-bet and STAT1 (Quirion et al. 2009).

Ikaros also promotes Th17 differentiation. A lack of Ikaros results in significantly reduced expression of Th17 effector cytokines as well as lineage determining factors including RORγt in IK-/- Th17 differentiation cultures (Wong et al. 2013). In Th17 cultures, Ikaros also functions to repress genes that limit Th-17 differentiation such as *Foxp3* and *Tbx21* (Wong et al. 2013).

Ikaros plays a direct role in activation and repression of cytokine genes. Its’ role in activation of *il10*, and repression of *il2* and *ifng* gene expression in CD4 T cells has been defined. IL-10 is a potent anti-inflammatory and immunosuppressive cytokine that inhibits DC and macrophage functions. IL-10 induces down-regulation of MHC class II and the costimulatory molecules CD80 and CD86 on APCs, in addition to repressing production of the proinflammatory cytokines (Moore et al. 2001). IK-/- T cells express significantly reduced levels of IL-10 (Umetsu and Winandy 2009). In addition, introduction of Ik-7, a dominant-negative Ikaros isoform, decreased IL-10 production in wild-type Th2 cells (Umetsu and Winandy 2009). Ikaros binds directly to *Il10* regulatory regions, suggesting a direct mechanism of regulation (Umetsu and Winandy 2009). In
Th2 cultures, Ikaros binds directly to the $\text{Ifn}_\gamma$ locus, reflecting a direct role in silencing of $\text{Ifn}_\gamma$ (Quirion et al. 2009).

IL-2 is essential for survival and proliferation of activated T cells. Interleukin-2 receptor $\alpha$ chain (IL-2R$\alpha$) expression is induced upon activation of T cells. Ikaros represses $il2$ gene expression in association with histone deacetylation at the $il2$ locus (Bandyopadhyay et al. 2007; Thomas et al. 2007). Following stimulation with anti-CD3 and anti-CD28, T cells from IK$^-/-$ mice express much higher levels of IL-2 than wild type T cells. siRNA knock down of Ikaros in wild type T cells led to increased production of IL-2 and resistance to T cell anergy.

In conclusion, Ikaros regulates multiple events in CD4 T cell differentiation and function including Th cell lineage fate decisions, cytokine gene expression and regulation of proliferative responses. However, the role of Ikaros in naïve CD4 T cells, is largely unknown.

### 1.3 FoxO Family of Transcription Factors

Prior to activation, naïve T cells are maintained in circulation for a long period of time without any antigenic stimulation (Jameson 2002). IL-7 is a cytokine which initiates signaling essential for T cell development in the thymus. IL-7 is also required for peripheral naïve T cell survival and is important in maintaining T cell homeostasis (Tan et al. 2001). In lymphopenic mice, mice lacking both B and T cells, self-MHC recognition and IL-7 signaling promote the proliferation of adoptively transferred naïve T cells to fill the niche (Tanchot et al. 1997). These studies have also led to the discovery of
specific factors that integrate environmental signals and regulate T cell survival and homeostasis (Takada and Jameson 2009; Jameson 2002).

*FoxO family of transcription factors in T cells*

The Forkhead box O (FoxO) family of transcription factors function by sensing physiological stimuli such as nutrients, growth factors, or stress and convert this information into programs of gene expression that regulate cell cycle progression, survival/apoptosis, metabolism and differentiation (Hedrick et al. 2012; Dejean et al. 2011) (Figure 3). FoxO factors are evolutionarily conserved from C. elegans to humans. DAF-16 is the sole ortholog of the FoxO family of transcription factors, influencing the rate of ageing of *Caenorhabditis elegans* in response to insulin/insulin-like growth factor 1 (IGF-I) signaling (A. T. Y. Chen et al. 2015). By translocating to the nucleus, DAF-16/FoxO factors regulate genes that result in delayed reproduction and growth while increasing resistance to stress, starvation tolerance and longevity (K. Lin et al. 1997; Henderson and Johnson 2001).

In mammals, the FoxO family of transcription factors is comprised of four members: Foxo1, Foxo3, Foxo4 and Foxo6. Foxo6 expression is confined to specific region of the brain (Jacobs et al. 2003). Foxo1 and Foxo3 are expressed in T cells, Foxo1 being expressed at higher levels. The roles of Foxo1 and Foxo3 have been analyzed in a variety of mouse tissues. Foxo3 is required for activation-induced cell death, and regulates cell cycle progression through transcriptional induction of *CDKN1B*, encoding the protein p27KIP1 (Dijkers et al. 2000; Van Der Heide et al. 2004). In mice, conditional
deletion of Foxo1 in T cells leads to detrimental effects on T cell homeostasis in vivo (Dejean et al. 2011). Several direct Foxo1 target genes have been identified in T cells. One such target is \( \text{Il7ra} \) which encodes IL-7R\( \alpha \), a subunit of the IL-7R, a receptor critical for naïve T cell survival (Kerdiles et al. 2009; Schluns et al. 2000). Foxo1 binds to an enhancer approximately 3 kb upstream of the \( \text{Il7ra} \) start site, and conditional deletion of Foxo1 causes a decrease in \( \text{Il7ra} \) expression in naïve T cells (Kerdiles et al. 2009). Decreased \( \text{Il}-7\text{ra} \) expression results in decreased survival of Foxo1-deficient CD4 T cells in response to IL-7.

A second important direct target of Foxo1 is \( \text{Sell} \), the gene encoding L-selectin (CD62L), an adhesion molecule expressed at high levels on the surface of naïve T cells. CD62L binding of ligand on the surface of high endothelial venules, leads to naïve T cell entry into lymph nodes. This is referred to as T cell “homing”. T cells with a conditional deletion of Foxo1 express less CD62L, resulting in reduced homing to lymph nodes (Kerdiles et al. 2009). Expression of \( \text{Sell} \) is dependent on another important transcriptional regulator \( \text{Klf2} \). \( \text{Klf2} \) is also a direct target of Foxo1. \( \text{Klf2} \) binds and activates the promoter for \( \text{S1P}1 \), a receptor that is critical for thymocyte egress and T cell recirculation (Gubbels Bupp et al. 2009; Kerdiles et al. 2009; Ouyang et al. 2009).
Figure 3. FoxO factor signaling, regulation and localization.

Depending on the type of environmental stimuli, FoxO factors undergo post-translational modifications that determine nuclear versus cytoplasmic localization of FoxO factors. In response to TCR stimulation, activation of the PI3K/Akt signaling pathway results in FoxO phosphorylation. Phosphorylation events downstream of PI3K/Akt signaling in response to growth factor binding or JNK signaling in response to oxidative stress, lead to nuclear export and degradation of FoxO proteins. Stress stimuli result in inhibition of PI3K–Akt signaling allowing for nuclear localization and activity of FoxO transcription factors. This inhibition directly involves the mTOR pathway. Pharmacological inhibition of mTOR via Rapamycin prevents FoxO phosphorylation and nuclear export. Nuclear retention of FoxO factors is important for upregulation of genes and factors important in development, metabolism, longevity and tumor suppression.
FoxO regulation

Regulation of FoxO activity can be controlled by posttranslational modifications. These modifications determine nuclear versus cytoplasmic localization of FoxO factors (Figure 2). T cell activation prompts phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMS) on the cytosolic side of the TCR/CD3 complex by the lymphocyte protein tyrosine kinase (Lck) (Cronin and Penninger 2007). Bound Lck acts as a docking site for phosphoinositide-3 kinase (PI3K). Recruitment of PI3K to the T cell receptor relocates PI3K to its lipid substrates, where it phosphorylates the 3'-hydroxy group of the inositol ring of phosphatidylinositol to generate PIP2 (phosphatidylinositol 4,5- bisphosphate) and PIP3 (phosphatidylinositol 3,4,5-trisphosphate) (Vanhaesebroeck and Alessi 2000). These two signaling molecules trigger downstream serine/threonine kinases that include protein kinase B (PKB), also known as Akt. Activated Akt then proceeds to phosphorylate serine/threonine residues on target proteins such as FoxO factors. Phosphorylation of FoxO factors causes their nuclear export and degradation (Van Der Heide et al. 2004), resulting in a shutdown of FoxO activity (Brunet et al. 2002).

FoxO transcription factors are re-localized to the nucleus in response to growth factor withdrawal, oxidative stress and nutritional availability, all conditions that determine the effectiveness of an immune response (Hedrick 2009). FoxO transcription factors regulate cell survival, proliferation and metabolism, and these activities of FoxO affect the expansion and contraction of antigen-responsive lymphocyte populations (Hedrick et al. 2012). In T cells, in response to cytokine/growth factor withdrawal, Foxo1
promotes recruitment of T cells to lymphoid organs for IL-7 mediated survival, through upregulation of Il7ra and Sell expression (Dejean et al. 2011).

Oxidative stress or nutrient deprivation can also counteract FoxO nuclear export. Oxidative stress induced by hydrogen peroxide causes phosphorylation of Foxo3 mediated by the kinase MST1 (Lehtinen et al. 2006). In addition, cells treated with hydrogen peroxide activate the small GTPase Ral resulting in JNK-dependent phosphorylation of Foxo4 (Essers et al. 2004). Such phosphorylation events disrupt interactions with 14-3-3 regulatory proteins and promote FoxO translocation to the nucleus. FoxO nuclear localization induces programs of gene expression involved in detoxification of reactive oxygen species (Hedrick 2009; Kops et al. 2002). This is demonstrated by the deletion of Foxo1, Foxo3 and Foxo4 in hematopoietic stem cells, which results in increased production of reactive oxygen species and lower stem cell longevity (Tothova et al. 2007). Under starvation conditions, FoxO factors are phosphorylated at two or more sites by AMP-activated protein kinase leading to induction of genes important for metabolism and stress resistance (Greer et al. 2007).

**Foxo1 and Regulatory T Cell Differentiation**

In addition to decreased cell survival and defective homing, Foxo1-deficient T cells display defects in Treg differentiation and function (Kerdiles et al. 2010). The expression of Foxp3 is important to Treg function as its absence leads to the onset of IPEX syndrome. The onset of a similar inflammatory disorder is seen in Foxo1 conditional knockout mice (Ouyang et al. 2009). In the absence of Foxo1 in Treg cells,
mice develop pathology that leads to death within 35 days of birth. A similar phenotype is seen in mice with the scurfy mutation of the Foxp3 gene, or mice depleted of Treg cells (Lahl et al. 2007). These studies prompted investigation into the mechanism of Foxo1-regulation of Foxp3 expression.

A proposed mechanism of Foxo1-mediated regulation of Foxp3 has been via TGF-β. In oligodendrocyte progenitor populations, TGF-β signaling led to Foxo1 and Smad3/4 interaction in transcriptional regulation of oligodendrocyte differentiation (Kerdiles et al. 2010; Palazuelos et al. 2014). This led to the investigation that Foxo1 might also be essential for TGF-β-induced differentiation of iTreg cells. A conditional deletion of Foxo1 in T cells resulted in decreased iTreg induction in the presence of TGF-β (Kerdiles et al. 2010). In addition, the majority of Foxo1-deficient T cells in iTreg differentiation cultures produce IFN-γ, accompanied by increased T-bet expression, as if they had been misdirected to become Th1 cells (Kerdiles et al. 2010).

Reports have suggested that Foxo1 plays a role in transcriptional control of Foxp3. Binding sites for Foxo1 have been identified within an intron of the Foxp3 locus (Ouyang et al. 2012; Ouyang et al. 2010).

Thus, Foxo transcription factors play an essential role in specifying the program of T cell differentiation, most importantly in the pathway leading to development and function of Treg cells.
1.4 Summary and Conclusions

An extensive network of transcription factors and cytokine signaling pathways is essential in creation of a T cell repertoire that is responsive to a wide array of antigens. Ikaros has been placed in this network of transcription factors. Beginning at the hematopoietic stem cell stage through differentiation into polarized T helper subsets, Ikaros is important in regulation of CD4 T cell lineage fate decisions and proliferative responses. However, the role of Ikaros in naïve T cells has not been explored. In this dissertation, I have shown that Ikaros plays an important role in maintaining the naïve CD4 T cell compartment through regulating expression of Foxo1. I have also demonstrated that Ikaros is essential for iTreg development.

The Foxo family of transcription factors have a significant role in immune system homeostasis – a process necessary in maintaining a pool of naïve, resting CD4 T cells in circulation. By sensing environmental stimuli such as nutrients, growth factors or stress, Foxo factors activate programs of gene expression that dictate proliferation, differentiation, survival and death of various cell types. One member of the Foxo family, Foxo1, regulates naïve T cell survival, homeostasis, homing and iTreg differentiation through direct regulation of corresponding genes encoding IL-7Rα, CD62L and Foxp3, respectively. Foxo1 expression is known to be regulated by posttranslational modifications that control subcellular localization and stability. However, little is known about how the gene encoding Foxo1 is transcriptionally regulated. Studies presented in this dissertation have revealed distinct yet overlapping roles of Ikaros and Foxo1 in regulation of programs of gene expression in naïve CD4 T cells.
Chapter 3 describes mechanisms by which Ikaros regulates CD4 T cell survival and homing, highlighting a potential role for Ikaros in regulating Foxo1 gene expression.

Chapter 4 reveals defects in iTreg differentiation in the absence of Ikaros. These findings will contribute towards defining a role for the Ikaros/Foxo1 axis in determination of CD4 T cell fate and homeostasis. These studies are also the first to identify Ikaros as a transcriptional regulator for Foxo1 gene expression in naïve CD4 T cells.
CHAPTER 2. MATERIALS AND METHODS

2.1 Mice

Ikaros null (IK/-) mice (C57BL/6 x SV129 and Balb/C background) were generated by intercrossing of IK/- heterozygotes. The phenotypes reported were consistent for both backgrounds. Foxp3/GFP reporter mice (Balb/C background) were gifted by Dr. Vijay Kuchroo at Harvard Medical School (Bettelli et al. 2006). Animals were bred and maintained in a specific pathogen-free barrier facility at Boston University School of Medicine. Genotypes were determined by PCR analyses. All animal procedures were approved by the Boston University Institutional Animal Care and Use Committee.

2.2 CD4 T Cell Purification

CD4+ or CD8+ T cells were isolated from pooled spleens of wild type or IK/- mice using CD4+ or CD8+ T Cell Isolation Kits (Invitrogen) following the Dynabeads protocol.

2.3 RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from the cells using the SV Total RNA Isolation System (Promega) and cDNA was generated with a Superscript III Kit (Invitrogen). Quantitative PCR was performed using iQ SYBR Green (Bio-Rad) and the BioRad MyiQ Real-Time PCR machine generating results that were analyzed using the Pfaffl method. Data are shown as ratios ((E{target})_{CT target}: (E{reference})_{CT reference}), where E = efficiency of PCR; target = gene of interest; and reference = HPRT. The
efficiencies of PCR reactions were calculated by analyzing the slope cycle threshold values generated in a standard curve, created using 10-fold dilutions of cDNA. Primers were synthesized by IDT DNA Technologies.

**Table 2. SYBR Green Quantitative Real Time PCR Primers.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hprt</em> forward</td>
<td>GGATATGCCCTTGACTATAATGAG</td>
</tr>
<tr>
<td><em>Hprt</em> reverse</td>
<td>GCCACAGGACTAGAACACC</td>
</tr>
<tr>
<td><em>Sell</em> forward</td>
<td>CCAAGTGTGCTTTCAACTGTTC</td>
</tr>
<tr>
<td><em>Sell</em> reverse</td>
<td>AAAGGCTCACACTGGACCAC</td>
</tr>
<tr>
<td><em>Il7ra</em> forward</td>
<td>ACCTGAAAGTCTTTATCGCAAAG</td>
</tr>
<tr>
<td><em>Il7ra</em> reverse</td>
<td>GTGGGATTGTGTCTTCTTGTTG</td>
</tr>
<tr>
<td><em>Foxy1</em> forward</td>
<td>ACGAGTGGATGTTGAAGAG</td>
</tr>
<tr>
<td><em>Foxy1</em> reverse</td>
<td>CGAATAAAGCTTGCTTGAAAGG</td>
</tr>
<tr>
<td><em>Foxy3</em> forward</td>
<td>AAGCAGACCCCTCAAACGTGACCGAA</td>
</tr>
<tr>
<td><em>Foxy3</em> reverse</td>
<td>AACGGATCAGTCCACCTTGCTGA</td>
</tr>
<tr>
<td><em>Foxy1SiteA</em> forward</td>
<td>GC GGCTTGAGTAGGAAT</td>
</tr>
<tr>
<td><em>Foxy1SiteA</em> reverse</td>
<td>CAATCGAGTACTCCAGGCA</td>
</tr>
<tr>
<td><em>Il2p</em> forward</td>
<td>TTCATAACGAAAGGGCTTCTTG</td>
</tr>
<tr>
<td><em>Il2p</em> reverse</td>
<td>GTGTGGCAGAAAGCATTACC</td>
</tr>
<tr>
<td><em>Foxy3</em> forward</td>
<td>GGCCTTCTCGAGGACAGA</td>
</tr>
<tr>
<td><em>Foxy3</em> reverse</td>
<td>GCTGATCATGGGTGCTGGTTGT</td>
</tr>
<tr>
<td><em>TGFBR1</em> forward</td>
<td>AGCATCACGGCCATCTGTG</td>
</tr>
<tr>
<td><em>TGFBR1</em> reverse</td>
<td>TGGCAAAACGTCTCCAGAGT</td>
</tr>
<tr>
<td><em>SMAD2</em> forward</td>
<td>ATGTCGTCCATCTTGCTTCC</td>
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<tr>
<td><em>SMAD2</em> reverse</td>
<td>AACCGTCCTGTATTCTTAGCTT</td>
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<td><em>SMAD3</em> reverse</td>
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<tr>
<td><em>Il21</em> forward</td>
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<tr>
<td><em>Il21</em> reverse</td>
<td>GGCCACGAGGTCAATGAT</td>
</tr>
<tr>
<td><em>Rorc</em> forward</td>
<td>ACCTCCACGTCCAGCTGCTG</td>
</tr>
<tr>
<td><em>Rorc</em> reverse</td>
<td>TCATTCTGCACTTCTGCTGACT</td>
</tr>
</tbody>
</table>
**2.4 Western Blots**

Protein extracts were prepared by whole cell lysis with Lysis Buffer (420 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 1% NP-40) supplemented with protease inhibitors. Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay. Lysates (15-20 mg total) were separated by gel electrophoresis on an 8% SDS-polyacrylamide gel and transferred to a PVDF membrane overnight at 4°C at 100 mA. Membranes were incubated with primary antibodies (Table 3) followed by horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse IgG) at 1:5000 for 1 hour at room temperature. Proteins were visualized by incubation with enhanced chemiluminescence reagent and exposure to film. ImageJ v1.43u (NIH) was used to measure intensity of bands.

**Table 3. Western blotting conditions.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name and Source</th>
<th>Isotype</th>
<th>Incubation</th>
<th>Exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxo1</td>
<td>C29H4, Cell Signaling</td>
<td>Rabbit</td>
<td>1:1000 O/N at 4°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Actin</td>
<td>A2066, Sigma</td>
<td>Rabbit</td>
<td>1:2500 O/N at 4°C</td>
<td>10sec/30sec</td>
</tr>
<tr>
<td>Akt</td>
<td>9272, Cell Signaling</td>
<td>Rabbit</td>
<td>1:1000 O/N at 4°C</td>
<td>3 min</td>
</tr>
<tr>
<td>pAkt</td>
<td>92715, Cell Signaling</td>
<td>Rabbit</td>
<td>1:500 O/N at 4°C</td>
<td>5-10 min</td>
</tr>
</tbody>
</table>
2.5 Annexin V Staining and Survival Assays

Purified CD4 T cells were plated in 96 well plates at 6.0x10^5 - 10^6 cells/well with or without 10ng/ml IL-7 (Peprotech). At 24, 48, and 72 hours post-plating, 2.0x10^5 cells were stained with Annexin V (Annexin V: PE Apoptosis Detection Kit 1, BD Biosciences) and analyzed by flow cytometry on a FACSCalibur (BD Biosciences) flow cytometer.

2.6 Flow Cytometry and Intracellular Staining

All antibodies were from eBioscience unless otherwise stated. The following antibodies were used for analyses of cell surface markers: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-H-2Kk (H100-27.R55) (Miltenyi Biotech), anti-Thy1.1 (HIS51), anti-IL-7Rα (SB/199), anti-CD62L (MEL-14) and anti-CD44 (IM7). For intracellular phospho-Akt analyses, spleens were dissociated directly into 10 ml of 1.6% paraformaldehyde (Electron Microscopy Sciences #15710). Cells sat at room temperature for 15 min followed by addition of 40ml 100% ice cold methanol to permeabilize cells. Cells were stained with anti-phospho Akt (pS473) antibody (M89-61, BD Biosciences). Intracellular staining for Foxo1, Foxp3 and Ki67 was performed using Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience). For Foxo1 staining, cells were stained first with anti-Foxo1 (Cell Signaling C29H4) followed by/anti-Rabbit IgG FITC (Fluorescein isothiocyanate) (Jackson ImmunoResearch). For Foxp3 staining, cells were stained with anti-Foxp3-PE (Phycoerythrin) (150D/E4). For Ki67 staining, cells were stained with anti-Ki67-PE (SolA15). Cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) flow cytometer. Analyses were performed using Flow Jo software.
2.7 CD4 T Cell Differentiation Cultures

CD4+ T cells purified from pooled spleens from two to four mice were stimulated in 12 well plates with 2 µg/mL plate-bound anti-CD3 (2C11) and 5 µg/mL soluble anti-CD28 (37.51) in RPMI 1640 medium supplemented with 10% FCS, 50 µM 2-ME, 200nM L-glutamine (Hyclone) and 100 U/mL of penicillin-streptomycin (RPMI complete). For iTreg differentiations, cells were grown in culture for 72 hours with 2 ng/ml TGF-β and 50U/ml IL-2 after being removed from anti-CD3 stimulation at 24 hours. Prior to flow cytometry analyses for time course experiment, 5µl of propidium iodide (PI) was added per tube, and PI negative gated cells were representative of the live cell population. For some iTreg differentiation cultures, anti-IFN-γ (XMG1.2) or 25nM rapamycin (Sigma) were added. Th1 differentiation was induced by culturing with 1 ng/ml IL-12 and 5 µg/ml anti-IL-4. Th17 differentiation was induced by adding 5 µg/ml IL-6, 1 ng/ml TGF-β, 5 µg/ml anti-IFN-γ and 5 µg/ml anti-IL-4. All cytokines were from Peprotech.

2.8 CD4 T Cell Purification

CD4+ T cells purified from wild type and IK-/− spleens were differentially labeled with 10 mM Efluor-450 (eBioscience) and 1 mM CFSE (eBioscience) respectively. Labeled cells were injected retro-orbitally (2x10^6 cells per genotype) into wild type hosts. Inguinal lymph nodes and spleens were harvested after 24 hours. Labeled cells in each organ were detected using a FACSCalibur flow cytometer (BD Biosciences).
2.9 Retroviral Transductions

The MSCV IRES H-2Kk and MSCV IRES Ik-1 H-2Kk constructs were generated and described in studies by Kathrein et al. (Kathrein et al. 2005). The MSCV Foxo1A3 Thy1.1 and MSCV Thy1.1 constructs were gifts from Dr. David Fruman (University of California, Irvine) (Yusuf et al. 2004). Retroviruses were produced via transfection of retroviral constructs into the Phoenix packaging cell line using a calcium phosphate transfection protocol. Viral supernatants were harvested at 24 hours post-transfection and 1ml was used to infect T cells that had been activated overnight with anti-CD3/anti-CD28. Supernatants were supplemented with 6 µg/ml polybrene. Plates were centrifuged at 1900 rpm for 2 hours at 32°C. Supernatants were removed and replaced with RPMI complete media. After 24 hours cells were removed from stimulation and supplemented with 50U/ml IL-2. After 48 hours, wells were replaced with fresh media without IL-2 and cells were rested for 72 hours followed by staining and analysis. Anti-H-2Kk PE (H-100-27.R55) or anti-Thy1.1 (H1S51) antibody were used to identify successfully transduced cells. All stained cell populations were detected using a FACSCalibur flow cytometer (BD Biosciences).

2.10 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed using chromatin prepared from purified wild type and IK-/− CD4 T cells using the ChIP assay kit (Millipore). 5 x 10⁶ cells were used per sample for cross-linking. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature to preserve protein-DNA interaction. The reaction was stopped by adding 104 µl/mL of 10x glycine. Cells were washed
with PBS and lysed with 500 mL of lysis buffer (Millipore) on ice for 10 min. Cell lysates of $5 \times 10^6$ cells were divided into aliquots of $1.67 \times 10^6$ cells for sonication. Cells were sonicated for 8 cycles of 5 min pulses (30 sec on and 5 sec off) to shear DNA in the cold room. Samples were precleared with protein G-agarose/salmon sperm beads (Millipore). Protein-DNA complexes were immunoprecipitated using the ChIP Assay Kit (Millipore) and antibodies against acetylated histone 3 (06-599B; Upstate Cell Signaling Solutions). Complexes were collected with Protein G agarose/salmon sperm beads and washed. Protein-DNA complexes were eluted off the beads and cross-links were reversed by heating at 65°C overnight. DNA was recovered by phenol-chloroform extraction and precipitated by ethanol. qPCR analyses were performed on immunoprecipitated DNA and normalized to total chromatin input using the Pfaffl method.

### 2.11 Statistics

Statistical analyses were performed using paired and unpaired two-tailed Student $t$ tests with the GraphPad Prism software. A two-way ANOVA with repeated measures analysis was performed for the cell survival assay, also using the GraphPad Prism software.
CHAPTER 3. IKAROS REGULATES FOXO1-DRIVEN PROGRAMS OF NAÏVE CD4 T CELL MAINTENANCE

3.1 Introduction

CD4 T cells play an important role in establishing adaptive immunity. Defects in CD4 T cell development lead to severe immunodeficiency and autoimmunity. An extensive network of cytokines and transcription factors regulate processes of T cell maturation and differentiation into effector subsets. Ikaros is one such factor. Ikaros has been proven to be essential in both early and late stages of CD4 T cell development, through regulation of genes important in determination of T helper cell fate and subset differentiation (Georgopoulos et al. 1994; Yoshida et al. 2006; Wong et al. 2013; Nichogiannopoulou et al. 1999; Georgopoulos et al. 1992). However, a role for Ikaros in maintaining a naïve CD4 T cell population has yet to be determined.

Mature CD4 T cells that do not encounter antigen remain in a “naïve” state (Takada and Jameson 2009). Survival, homeostatic proliferation and homing of these cells is crucial, as this cell pool contributes numbers of CD4 T cells available for antigen encounter (Kieper and Jameson 1999). One mechanism by which a naïve T cell is maintained in the periphery is via up-regulation of IL-7Rα on its cell surface. IL-7R engagement initiates signaling cascades essential for homeostatic proliferation and cell survival (Schluns et al. 2000; Tan et. al 2001). Proper homing of naive T cells to peripheral lymphoid organs is dependent on cell surface expression of L-selectin, or CD62L (Kerdiles et al. 2009). CD62L interacts with ligands expressed on the surface of
high endothelial venules (HEVs), initiating T cell extravasation into lymph nodes from
the bloodstream (Arbonés et al. 1994).

IL-7Rα and CD62L are molecules that are regulated by the transcription factor Foxo1
Belonging to the FoxO family of transcription factors, Foxo1 is most highly expressed in
T cells (Hedrick et al. 2012; Kerdiles et al. 2009). Conditional deletion of Foxo1 severely
affects naïve T cell homeostasis due to decreased survival in the periphery (Kerdiles et al.
2009). Foxo1-deficient T cells also do not home to lymph nodes (Kerdiles et al. 2009). In
this study we show that IK-/- CD4 T cells also display similar defects, which led to the
investigation of Ikaros’ role in coordination of Foxo1 activity.

In this chapter, a role for Ikaros is revealed in maintaining programs of naïve CD4
T cell survival and homing. Mechanisms reveal Ikaros as a potential regulator of Foxo1
in naive CD4 T cells. In comparison to wild-type, IK-/- CD4 T have decreased survival in
response to IL-7. Differential CD4 T cell homing is also observed, where IK-/- CD4 T
cells display decreased homing to lymph nodes. We show that abatement in survivability
and homing to lymph nodes is likely due to decreased expression of IL-7Rα and CD62L
respectively. Furthermore, reduced expression of genes encoding IL-7Rα and CD62L,
\( il7ra \) and \( Sell \), is also observed, which can be linked to low levels of Foxo1 mRNA and
protein in the absence of Ikaros. Re-introduction of Ikaros into IK-/- CD4 T cells cultures
via retroviral transduction increases Foxo1 mRNA, resulting in upregulation of IL-7Rα
expression. Re-introducing Foxo1 into IK-/- CD4 T cells results in increased CD62L
expression. Ikaros therefore plays a differential role in regulation of Foxo1 and its targets.
3.2 Ikaros is Required for Survival and Homing of Naïve CD4 T Cells

Ikaros is expressed at high levels within the thymus, regulating gene expression in T cells and their progenitors (Georgopoulos et al. 1992). Ikaros is also highly expressed in differentiating CD4 T lymphocytes functioning as a transcriptional activator and repressor in regulation of key cytokines and downstream differentiation factors (Georgopolous et al. 1997; Quirion et al. 2009; Wong et al. 2013). In order to investigate a potential role for Ikaros in the naïve T cell, we tested the survival and homing capabilities of naïve CD4 T cells that lack Ikaros. For these studies, IK-/– mice were used. These mice harbor a deletion in exon 7 of the Ikaros gene, and as a result lack Ikaros protein (Wang et al. 1996). To assess survivability, CD4 T cells were purified from spleens of IK-/– and wild type mice and were plated with or without exogenous IL-7. Cell viability was measured at 24 hour intervals via staining with fluorescently-conjugated Annexin V, a peptide that binds to externalized phosphatidylserine (PS) on the surface of apoptotic cells. In the absence of IL-7, both IK-/– and wild type CD4 T cell populations survive poorly over time (Figure 4A). However, in the presence of IL-7, wild type CD4 T cells were able to recover their ability to survive, whereas IK-/– CD4 T cells could not (Figure 4B). This data suggests that cells lacking Ikaros have a decreased ability to survive in response to IL-7 pro-survival signaling mechanisms.

Along with being able to survive in the periphery, it is important that a naïve CD4 T cell be able to home to lymph nodes (Itano and Jenkins 2003). Studies using adoptive transfer experiments in irradiated mice have established that transferred T cells have the
capacity to proliferate and occupy space within the spleens and lymph nodes of immunodeficient hosts (Tanchot et al. 1997). Adaptations of these adoptive transfer experiments were implemented in order to study the role of Ikaros in CD4 T cell homing in wild type mice, as IK-/- mice do not have lymph nodes (Georgopoulos et al. 1994; Wang et al. 1996). In the first set of experiments, both purified IK-/- and wild type CD4 T cells were loaded with Carboxyfluorescein succinimidyl ester (CFSE), an intracellular fluorescein staining dye. Staining was followed by retro-orbital injection of labeled cells into two separate wild type hosts. IK-/- CD4 T cells displayed differential homing capabilities, with a reduced ability to traffic to lymph nodes and preferential homing to the spleen (Figure 5A). Next, to erase the potential factor of injection efficiency discrepancies between mice hosts, IK-/- and wild type T cells were differentially loaded with CFSE and Efluor 450, a violet fluorescent intracellular dye, and transferred in a 1:1 ratio into the same wild type host. In agreement with the previous experiment, significant reduction in the ability of IK-/- CD4 T cells to traffic to lymph nodes was observed (Figure 5B). These results suggest an important role for Ikaros in allowing for proper homing of naïve CD4 T cells to peripheral lymph nodes.

Taken together, these data demonstrate a role for Ikaros in regulating survival and homing of naïve CD4 T cells.
Figure 4. IK-/- CD4 T cells display defects in survival.

A) Cell viability assay for purified wild type (solid) and IK-/- (dotted) CD4 T cells in culture without exogenous IL-7, measured as percent of cells that stained negative for Annexin V (N=2). B) Cell viability assay for purified wild type (solid) and IK-/- (dotted) CD4 T cells in culture with 10ng/ul of exogenous IL-7, measured as percent of cells that stained negative for Annexin V (N=4). (*p<0.05 for wild type versus IK-/-).
3.3 Decreased Expression of *Sell* and *il7ra* in IK-/- CD4 T cells

Observed defects in survivability and homing of naïve CD4 T cells in the absence of Ikaros, led to investigation of mechanisms by which Ikaros regulates these processes. Flow cytometry was used to measure cell surface expression of CD62L and IL-7Rα, followed by quantitative real-time polymerase chain reaction analyses (qRT-PCR) for analyzing expression of corresponding genes *Sell* and *Il7ra*, respectively. Significant reduction in CD62L and IL-7Rα expression on the surface of IK-/- CD4 T cells (Figure 6A) was accompanied by reduced expression of both genes (Figure 6B). IK-/- CD8 T cells also displayed significantly reduced levels of expression of *Sell* and *Il7ra* (Figure 6C). These data suggest that Ikaros, through regulation of key cell surface molecules IL-7Rα and CD62L, is essential for survival and homing of naïve T cells.

Lower levels of CD62L expression are associated with activated and memory T cell populations (Jung et al. 1988), and levels of IL-7Rα are transiently reduced at early stages of T cell activation (Park et al. 2004). Therefore, decreased expression of CD62L and IL-7Rα observed in IK-/- CD4 T cells may be a result of an expanding activated or memory cell population. To test for this, levels of CD44 and CD25 were examined as increased expression of these markers occurs on activated and/or previously activated T cells (Puré and Cuff 2001). Freshly isolated IK-/- CD4 T cells did not exhibit increased CD44 expression (Figure 7A). In fact, levels of CD44 were significantly lower than observed on their wild type counterparts (Figure 7B). CD25 is the alpha chain of the IL-2R, which is expressed on activated T cells. Cell surface CD25 expression on freshly
isolated as well as activated IK-/− CD4 T cells was not increased relative to wild-type levels. These experiments were followed by intracellular staining analysis of Ki67 expression. Ki67 is a cell marker for cell proliferation and is only expressed in activated cells, undergoing cell division (Scholzen and Gerdes 2000). Measuring Ki67 levels was therefore essential in confirming a lack of cell proliferation. Flow cytometry data reveal that there were no differences in levels of Ki67 expression in freshly isolated wild-type and IK-/− CD4 T cells. In addition, CD44, CD25 and Ki67 expression was measured in activated cells, as controls. These results show that IK-/− cells are also able to upregulate expression of these markers once activated, just as wild type cells. Not only do these results confirm that IK-/− CD4 T cells are not proliferating or activated, but provide evidence that they are in a naïve state.

Lastly, levels of CD62L and IL-7Rα expression were analyzed in IK-/− CD4 SP thymocytes. Cells freshly isolated from the thymus represent a population that have not undergone any peripheral phenotypic alterations due to activation or proliferation signals (Takada and Jameson 2009). IK-/− CD4 T cells from the thymus were also CD62Llo/IL-7Rαlo/CD44lo, providing evidence that they acquire this phenotype in the thymus (Figure 7C). Taken together, these data support a developmental role for Ikaros in regulation of genes encoding IL-7Rα and CD62L, rather than an expansion of activated/memory subsets once IK-/− CD4 T cells exit into the periphery.
Figure 5. A lack of Ikaros results in decreased naïve CD4T cell homing to peripheral lymph nodes.

A) Differentially labeled wild type (Efluor 450) and IK-/- (CFSE) CD4 T cells were adoptively transferred in two separate wild-type hosts. (N=2) % Recovery=Individual percentage of wild type (Efluor 450) or IK-/- (CFSE) cells in the spleen or lymph node/total percentage of labeled (Efluor 450 +CFSE) cells in the spleen or lymph node. B) Differentially labeled wild type (Efluor 450) and IK-/- (CFSE) CD4 T cells were adoptively transferred in a 1:1 ratio into the same wild type mouse. (For spleen N=3, for LN N=3, *p=0.0501 **p<0.01).
Figure 6. IK-/- cells display decreased expression of molecules IL-7Rα and CD62L which are important in survival and homing.

A) Flow cytometry analyses measuring expression of CD62L and IL-7Rα on the surface of CD4 splenic T cells from wild type (dark shaded histogram) and IK-/- (black outlined histogram) mice in comparison to an isotype control (light shaded histogram). The bar graph is representative of mean fluorescence intensities compiled from three experiments (**p<0.01,*p<0.05). B) qRT-PCR analyses of Sell and Il7ra expression using cDNA from wild type and IK-/- purified CD4 T cells. Values were normalized to levels of expression of the housekeeping gene, HPRT, for a minimum of six individual experiments. (**p<0.001). C) qRT-PCR analyses of Sell and Il7ra expression using cDNA from wild type and IK-/- purified splenic CD8 T cells. Values were normalized to levels of expression of the housekeeping gene, HPRT, for a minimum of five individual experiments. (**p<0.01, ***p<0.001).
Figure 7. IK-/- CD4 T cells do not represent an activated or memory cell population.

A) Flow cytometry analyses of CD44, CD25 and Ki67 expression in purified wild type (dark shaded histograms) and IK-/- (black outlined histograms) freshly isolated and activated CD4 T cells in comparison to isotype controls (light shaded histograms). Data is representative of two independent experiments. B) Compilation of mean fluorescence intensities of CD44 cell surface expression in purified wild type (black) and IK-/- (black lined) splenic CD4 T cells (N=3, **p<0.01) C) Flow cytometric analyses of CD62L, IL-7Rα and CD44 expression in wild type (dark shaded histogram) and IK-/- (black outlined histogram) CD4 single positive T cells in the thymus in comparison to isotype control (light shaded histogram). Bar graph is representative of mean fluorescence intensities compiled from three experiments (*p<0.05,**p<0.01,***p<0.0001).
3.4 Reduced Expression of Foxo1 in the Absence of Ikaros

Ikaros has been shown to regulate multiple processes of CD4 T cell development. We have also described a role for Ikaros in regulation of survival and homing of naïve CD4 T cells in the periphery. Foxo1, has been identified as an important factor in regulation of naïve T cell homeostasis, directly activating expression of both Il7ra and Sell genes (Hedrick et al. 2012; Kerdiles et al. 2009; Ouyang et al. 2009). Interestingly, Foxo1 deficient CD4 T cells display similar survival and homing defects as those observed in IK-/- T cells (Kerdiles et al. 2009). Foxo1 activity in the absence of Ikaros, could therefore also be altered in naïve T cells. Western blot analyses demonstrate a significant reduction in levels of Foxo1 protein in splenic IK-/- CD4 T cells (Figure 8A). Intracellular staining for Foxo1 supported this in both CD4 and CD8 T cells from IK-/- spleens (Figure 8B). This suggests, that a possible mechanism by which Ikaros regulates Il7ra and Sell is through regulation of Foxo1 expression.

Foxo1 protein levels are, in part, controlled post-translationally through the PI3K/Akt signaling pathway (Van Der Heide et al. 2004; Brunet et al. 2002). IK-/- T cells may have increased levels of phosphorylated, active Akt (p-Akt), which could lead to Foxo1 phosphorylation causing its export from the nucleus and subsequent degradation. To examine if this is the mechanism underlying the defect in Foxo1 expression in IK-/- CD4 T cells, levels of p-Akt were compared to wild type by Western blot and intracellular flow analyses (Figure 9A, 9B). Both methods show comparable levels of p-Akt in IK-/- and wild-type CD4 T cells, suggesting that elevated levels of active p-Akt are not responsible for decreased Foxo1 expression in cells lacking Ikaros.
Ikaros may also regulate Foxo1 at the level of transcription. QRT-PCR analysis indicated that levels of Foxo1 mRNA are significantly reduced in the absence of Ikaros (Figure 10). In contrast levels of Foxo3 mRNA are equivalent in IK-/- and wild type CD4 T cells.

These studies confirm a role for Ikaros in regulation of Foxo1 expression, however mechanisms of regulation still need to be explored.
Figure 8. In the absence of Ikaros, decreased levels of Foxo1 protein are observed in T cells.

A) Western blot analyses of Foxo1 expression in purified splenic CD4+ T cells from wild type and IK-/- mice. Intensity of bands for Foxo1, normalized to actin bands, is shown as fold over wild type levels below each band. Average of relative intensity of Foxo1 in Ikaros null compared wild type T cells over three independent experiments are compiled in bar graph. B) Levels of Foxo1 in CD4 and CD8 T cells in wild type (shaded histogram) and IK-/- (black outlined histogram) spleens as determined by intracellular staining followed by flow cytometric analyses.
Figure 9. Decreased levels of Foxo1 in IK-/- CD4 T cells is not due to increased levels of activated Akt.

A) Akt and phospho-Akt (p-Akt) expression levels were analyzed by Western blot using whole-cell lysates prepared from sorted CD4 T cells from spleens of wild type or IK-/- mice. The intensity of the bands for Akt and p-Akt are normalized to actin and shown as fold over wild type levels below each image. B) Levels of anti-phospho-Akt in CD4 T cells in wild type (black line) and IK-/- (light grey line) spleens as determined by intracellular staining followed by flow cytometric analyses.
Figure 10. Decreased expression of Foxo1 mRNA in the absence of Ikaros.

QRT-PCR analyses performed using cDNA from wild type and IK-/- purified splenic CD4+ T cells. (For Foxo1 n=9, *p<0.05. For Foxo3 n=7, p=0.3407, N.S.)
3.5 Effects of Lack of Ikaros on Up-regulation of the Foxo1 Program in Response to Stress

A consequence of TCR activation is the onset of PI3K/Akt signaling (Xue et al. 2008). While cells are resting, the PI3K/Akt pathway is not active. Foxo1 then remains in the nucleus, upregulating expression of its target genes (Van Der Heide et al. 2004). In response to cytokine/growth factor withdrawal, Foxo1 promotes recruitment of T cells to lymphoid organs for IL-7 mediated survival (Akhand et al. 2002). Foxo1-deficient CD4 T cells, cultured under conditions of cell stress, without added growth factors, are unable to upregulate levels of IL-7Rα and CD62L (Kerdiles et al. 2009). This assay was recapitulated using IK-/−and wild type CD4 T cells to test if Foxo1 target gene expression can be upregulated in the absence of Ikaros.

Purified wild type and IK-/− CD4 T cells were cultured without any TCR stimulation or added growth factors. After 24 hours, cell surface expression of IL-7Rα and CD62L was analyzed via flow cytometry, in comparison to freshly isolated cells. As observed in Foxo1-deficient CD4 T cells, IK-/− CD4 T cells could not upregulate expression of CD62L or IL-7Rα when rested overnight (Figure 11A). In fact, cell surface levels of IL-7Rα were significantly decreased. In addition, IK-/− CD4 T cells could not restore CD62L or IL-7Rα expression to the levels of wild type (Figure 11B). These data suggest that levels of Foxo1 are severely reduced in the absence of Ikaros. Even under conditions favoring Foxo1 nuclear retention and transcriptional activity, IK-/− CD4 T cells could not increase or restore expression of Foxo1 targets.
Figure 11. A lack of Ikaros compromises expression of IL-7Rα and CD62L, even under Foxo1-favoring, cell stress conditions.

A) Compilation of mean fluorescence intensities of three experiments measuring cell surface expression levels of CD62L and IL-7Rα in splenic purified wild type and IK−/− freshly isolated (black) CD4 T cells as well as cells that have been grown in the absence of growth factors overnight (dashed). (N=3; ***p<0.001,**p<0.01). B) Compilation of mean fluorescence intensities of three experiments measuring cell surface expression levels of CD62L and IL-7Rα in splenic purified wild type (grey) and IK−/− (white) CD4 T cells that have been cultured in the absence of growth factors overnight. (N=3; **p=0.0057,*p<0.05).
3.6 Interfering with Ikaros Activity has the Same Effect as Foxo1 Knockdown on Foxo1 Target Gene Expression

So far we have established that a lack of Ikaros causes decreased expression of CD62L and IL-7Rα. However, these decreases were observed in IK-/- cells, notorious for possessing multiple developmental defects. We therefore wanted to test if knocking down expression of either Ikaros or Foxo1 would result in similar defects in a wild type cell. Retroviral transductions were performed, as this is the most efficient method to deliver DNA to primary mouse T cells. A dominant negative Ikaros isoform, Ik-7 (MSCV IRES Ik-7 H-2Kk), and a Foxo1 shRNA (LMP Foxo A1 IRES GFP) were expressed in wild type CD4 T cells. H2K and GFP were co-expressed, allowing easy identification of successfully transduced cells. Ik-7 is a non-DNA binding Ikaros isoform that exerts a dominant negative effect, interfering with normal Ikaros activity (Morgan et al. 1997). Artificial small hairpin RNAs (shRNA), provide long-term silencing by targeting specific mRNAs for destruction. Wild type cells transduced with Foxo1-A1 acted as a positive control as the Foxo1 shRNA should decrease expression of CD62L and IL-7Rα. However, retroviral transduction requires activating T cells to induce proliferation for effective viral integration into the host genome. Upon induction of TCR signaling pathways, Foxo1 is phosphorylated and exported out of the nucleus (Van Der Heide et al. 2004). Therefore, critical resting protocols were implemented post transduction in order to drive nuclear localization of Foxo1, which is required for its function. It was even more important to restore a resting state as we wanted to attribute decreased expression of Foxo1 targets to decreased Ikaros, not to effects of activation. Cells were activated
overnight using anti-CD3/anti-CD28, followed by transduction. They were removed from stimulation 24 hours later. IL-2 was added to cultures to facilitate expansion and proliferation of transduced cells, allowing expression of IK-7 and the Foxo1 shRNA. Cultures were expanded for a period of 48 hours after which they were removed from IL-2, and plated with media with no added growth factors for 72 hours to induce a resting state before analysis.

IK-7 reduced cell surface expression of Foxo1 targets CD62L and IL-7Rα in wild type cells to nearly the same degree as Foxo1 shRNA (Figure 12). These results confirm that defects in expression of Foxo1 targets is due to a lack of Ikaros, not due to defective expression by an abnormal cell population. How Ikaros is regulating these targets still needs to be determined.

3.7 Ikaros Regulates Foxo1 Target Gene Expression Using Two Different Mechanisms

The decreased expression of CD62L and IL-7Rα in IK-/- T cells could be due to a direct role for Ikaros in their regulation, a role for an unknown Ikaros target gene in their expression or due to the decrease in Foxo1 observed in the absence of Ikaros. In order to place Foxo1 downstream of Ikaros in regulating expression of CD62L and IL-7Rα, we tested if restoring Ikaros or Foxo1 expression to IK-/- T cells could increase their expression. Retroviral transduction experiments were performed to determine whether Ikaros upregulation of Foxo1 target genes was dependent on Foxo1. These experiments involved introduction of Ikaros (MSCV IRES Ik-1 H2K) and Foxo1 (MSCV Foxo1 Thy1.1) gene constructs into purified wild type and IK-/- CD4 T cells. Successfully
transduced cells were identified via staining with fluorescently-conjugated anti-H2K and anti-Thy1.1 antibodies followed by flow cytometry analyses.

Re-introducing Ikaros into IK-/- CD4 T cells led to increased expression of IL-7Rα, but not CD62L. On the other hand, re-introduction of Foxo1 increased expression of CD62L, but not that of IL-7Rα (Figure 13). These results highlight a differential regulatory model for Ikaros in regulation of Foxo1 targets (Figure 14). We have previously shown that interference with Ikaros or Foxo1 activity via Ik-7 or Foxo1 shRNA, respectively, in wild-type CD4 T cells causes decreases in expression of CD62L and IL-7Rα. These data demonstrate that both Ikaros and Foxo1 are required to maintain their high level expression. Since only transduction of Ikaros into IK-/- CD4 T cells, which we hypothesize would also increase expression of Foxo1, was able to up-regulate IL-7Rα expression, this suggests that Ikaros and Foxo1 are both needed to co-regulate expression of the il7ra gene. 56% of Foxo1 target genes are also bound by Ikaros in pre-B cells, suggesting that they share targets (Ferreiros-Vidal et al. 2013).

In the case of CD62L, transduction of Foxo1 into IK-/- CD4 T cells was able to increase expression, suggesting that increasing Foxo1 expression could bypass lack of Ikaros. Therefore, Ikaros is required only to upregulate expression of Foxo1, and Foxo1 is able to regulate Sell expression without Ikaros co-regulation. It is puzzling that Ikaros transduction could not increase CD62L expression. This could possibly be explained by an altered ratio of Ikaros/Foxo1 levels within the transduced cell. Elevated levels of Ikaros may interfere with Foxo1's ability to bind and activate the Sell locus.
Figure 12. Interference with Ikaros and Foxo1 knockdown in wild type cells has similar effects on expression of Foxo1 targets.

Purified CD4 T cells from spleens of wild type and IK-/− mice were transduced with retroviruses prepared using MSCV IRES H-2Kk (MSCV), MSCV IRES H-2Kk IK-7 (IK-7) and Foxo1 shRNA (Foxo1-A1) constructs. Results are presented as a compilation of mean fluorescence intensities for CD62L and IL-7Rα expression in successfully transduced cells (H2K+, or GFP+) from two independent experiments, normalized to expression levels in control (MSCV) transduced cells.
Figure 13. Determining rescue effects on Foxo1 target gene expression by restoring Ikaros and Foxo1 gene expression.

Purified CD4 T cells from spleens of wild type and IK-/- mice were transduced with retroviruses prepared using MSCV IRES H-2Kk (MSCV-black), MSCV IRES Ik-1 H-2Kk (IK-1-grey) and MSCV Foxo1 Thy1.1 (Foxo1-white) constructs. Results are presented as a compilation of mean fluorescence intensities for CD62L and IL-7Rα expression from three independent experiments, normalized to expression levels in control (MSCV) transduced cells. For CD62L expression *p=0.05 for IK-/- Foxo1 in comparison to wild type and for IL-7Rα expression *p<0.05 for IK-/- IK-1 in comparison to wild type.
A) Ikaros upregulates expression of the Foxo1 gene. Ikaros and Foxo1 proteins bind to the *il7ra* locus, acting as co-regulators for *il7ra* gene activation. B) Ikaros upregulates expression of the Foxo1 gene. Foxo1 protein binds to the *Sell* locus, acting alone in *Sell* gene activation.

**Figure 14. Model representation of Ikaros regulation of Foxo1 targets.**
3.8 Ikaros Regulates Foxo1 Expression at the Level of Transcription

Next, we wanted to investigate mechanisms by which Ikaros may lead to increased expression of Foxo1. We have decreased expression of Foxo1 mRNA in the absence of Ikaros. This indicates a potential mechanism of Ikaros control of Foxo1 transcription. If Ikaros controls Foxo1 expression, restoring Ikaros expression to IK-/CD4 T cells should increase levels of Foxo1 message. In three independent retroviral transduction experiments, transduction of Ikaros (MSCV IRES Ik-1 H2K) increased expression of Foxo1 in both IK-/- and wild type CD4 T cells (Figure 15A).

Ikaros binds Foxo1 in pre-B cells and hematopoietic stem cells (Ferreiros-Vidal et al. 2013; Ng et al. 2009), suggesting a direct mode of regulation. This led us to investigate whether this were the case in CD4 T cells. A primer set was designed to span a region (Site A) of the Foxo1 locus, where Ikaros is known to bind in pre-B cells. We first attempted chromatin immunoprecipitation (ChIP) experiments using anti-Ikaros antibodies with chromatin prepared from freshly isolated wild-type CD4 T cells, in attempt to reveal direct binding of Ikaros to the Foxo1 locus. However numerous attempts with various monoclonal and polyclonal antibodies did not prove effective, as enrichment for Ikaros binding to the il2 promoter, a gene to which Ikaros directly binds and represses, was not observed. This led to us to conclude that the currently available Ikaros antibodies were not well suited for our experiments. Ikaros acts as an activator and repressor in transcriptional regulation of genes via association with chromatin remodeling complexes. Recruitment of histone acetyltransferases and deacetylases post-translationally modify histones, such as histone H3 (H3), to either permit or prevent
transcription, respectively. Ikaros binding has been shown to be associated with alterations in acetylation status of histones (Quirion et al. 2009; Thomas et al. 2007).

Therefore, we next wanted to test if, in the absence of Ikaros, acetylation status of histone H3 within the Foxo1 locus was altered. Chromatin was prepared from freshly isolated wild type and IK-/- CD4 T cells. ChIPs were performed using anti-acetylated histone 3 antibody (AcH3) and an antibody against histone H3, for normalization purposes. qRT-PCR results indicate decreased AcH3 levels within the Site A region of the Foxo1 locus in IK-/- CD4 T cells in comparison to wild type (Figure 15B). Importantly, acetylation status of the il2 promoter was used as a control, as Ikaros represses Il2 (Quirion et al. 2009; Bandyopadhyay et al. 2007). AcH3 levels at the Il2 promoter were increased in IK-/- CD4 T cells relative to wild-type as expected. These data suggest that in the absence of Ikaros, there is decreased accessibility at the Foxo1 locus, resulting in less recruitment of histone acetyltransferases and decreased Foxo1 gene transcription.
Figure 15. Ikaros regulates Foxo1 gene expression.

A) Purified CD4 T cells from spleens of wild type and IK-/- mice were transduced with retroviruses prepared from the MSCV IRES H-2Kk construct (MSCV) or the MSCV IRES Ik-1 H-2Kk construct (IK-1). Results of qRT-PCR analyses for Foxo1 expression are shown. Results have been normalized to expression levels in control (MSCV) transduced cells and results from three independent experiments are shown (**p<0.005). B) Antibodies against acetylated histone 3 (AcH3) were used for the ChIP assay. Primers were designed to amplify a region along the Foxo1 locus known to bind Ikaros (Foxo1SiteA). Il2 promoter primers were used as a positive control. Relative enrichment to input for AcH3 binding was obtained using qRT-PCR values that were normalized to levels of total chromatin input. These values were then analyzed using the following ratio= expression of acetylated histone 3/input: expression of histone 3/input for both wild type (black) and IK-/- (dashed) CD4 T cell chromatin.
3.9 Summary and Conclusions

Ikaros is important in regulation of CD4 T cell lineage fate decisions and proliferative responses. In this chapter, a role for Ikaros in regulation of naïve CD4 T cell survival and homing is revealed. Cell surface expression of genes encoding IL-7Rα and CD62L are crucial for allowing naïve T cells to survive and home to lymph nodes. In the absence of Ikaros, naïve CD4 T cells show decreased survival in culture, in the presence of IL-7. IK-/- CD4 T cells also display impaired migration to lymph nodes.

In support of its role in maintaining naïve T cell homeostasis, Foxo1 directly regulates expression of IL-7Rα and CD62L (Kerdiles et al. 2009). Our studies revealed that deficiencies in Foxo1 and Ikaros expression have similar consequences on T cell homeostasis, proliferation, survival and homing. This led to investigation of a potential regulatory role of Ikaros on Foxo1 activity. Foxo1 protein and mRNA levels in the absence of Ikaros are significantly decreased. Foxo1 regulation occurs at the protein level via posttranslational mechanisms. TCR activation turns on PI3K/Akt signaling. Akt phosphorylation of Foxo1 causes nuclear export and degradation of the protein (Van Der Heide et al. 2004). IK-/- cells could express elevated levels of p-Akt. Western blot analyses and intracellular staining results indicated similar levels to wild type, therefore ruling out this as a factor contributing to decreased Foxo1 levels.

Having established a potential regulatory role of Ikaros on Foxo1 expression, this mechanism had to be explored. Interfering with Ikaros and Foxo1 activity, via Ik-7 and Foxo1-A1 shRNA transduction respectively, resulted in similar decreases in expression of both targets. Having performed transduction using wild type cells allowed us to
confirm results that defects in expression of Foxo1 targets are due to a lack of Ikaros, not due to an abnormal IK-/- cell population.

These experiments were followed by rescuing Foxo1 expression in IK-/- CD4 T cells and examining the effect on expression of CD62L and IL-7Rα. Culturing CD4 T cells under non-stimulating, stress-inducing conditions, allowed for Foxo1 nuclear retention as the PI3K/Akt signaling pathway is shut off (Van Der Heide et al. 2004; Kerdiles et al. 2009). In such resting conditions, IK-/- CD4 T cells were unable to upregulate expression of Foxo1 targets. This supports the idea that IK-/- CD4 T cells have very low levels of Foxo1, as culturing cells under stress conditions in favor of Foxo1 nuclear retention and activity were not enough to overcome the defect. Re-introducing either Foxo1 or Ikaros gave us more specific insight into the differential regulation by Ikaros on expression of CD62L and IL-7Rα. Ikaros, but not Foxo1, transduction led to increased IL-7Rα expression whereas Foxo1, but not Ikaros, transduction led to increased CD62L levels in IK-/- CD4 T cells. These results indicated a preferential need for Ikaros and Foxo1 co-regulation for IL-7Rα expression. On the other hand, transduction of Foxo1 was able to increase CD62L expression alone, suggesting that it could bypass the need for Ikaros. In fact, increased Ikaros may interfere with Foxo1 regulation of CD62L.

Lastly, qRT-PCR analyses for Foxo1 mRNA expression, revealed increased levels of expression with Ikaros transduction. ChIP analysis of acetylated histone 3, along a region of the Foxo1 gene locus known to bind Ikaros in pro-B cells and HSCs (Scholzen et al. 2000; Xue et al. 2008) revealed decreased acetylation in IK-/- CD4 T cells in comparison to wild type. This confirms that in the absence of Ikaros, there is less...
Foxo1 gene activation.

In conclusion, studies presented in this chapter, have defined a novel Ikaros/Foxo1 transcriptional axis in regulation of naïve CD4 T cell survival and homing. They have also established the first insight into how the Foxo1 gene in transcriptionally regulated in the naive T cell.
CHAPTER 4. LACK OF IKAROS CAUSES DEFECTS IN REGULATORY T CELL DIFFERENTIATION

4.1 Introduction

Regulatory T cells (Tregs) represent an immunosuppressive T cell subset that function to down-regulate an immune response and maintain self tolerance, thereby preventing the onset of autoimmunity (Bloom et al. 1992; Groux et al. 1997; Chen et al. 1994). Development and function of the natural (nTreg) and induced (iTreg) subsets is dependent on expression of the master transcriptional regulator, Foxp3 (Hori et al. 2003; Gavin et al. 2007; Fontenot et al. 2003). nTregs represent a thymus-derived cell population important in maintaining immune tolerance and homeostasis (Miyara and Sakaguchi 2007; Sakaguchi et al. 1995). iTregs, also originating in the thymus, switch on Foxp3 expression in the periphery, controlling immunity at sites of inflammation (W. Chen et al. 2003). Anti-inflammatory properties of both nTregs and iTregs can be attributed to secretion of IL-10 and TGF-β, suppressing effector function of T cells and DCs and maintaining tolerance, respectively (Moore et al. 2001; de Waal Malefyt et al. 1991; Kriegel et al. 2006). Mutations in Foxp3 result in severe immune dysregulation in both humans and mice (Bennett et al. 2001; Lahl et al. 2007; Wildin et al. 2002; Brunkow et al. 2001).

TGF-β and IL-2 are important iTreg differentiation cytokines that activate Foxp3 gene expression (Sakaguchi et al. 1995; Rudensky et al. 2009; Marie et al. 2005; Fantini et al. 2004; Li et al. 2006). Signaling via the TGF-βR1 and TGF-βR2 complex involves phosphorylation of both transmembrane receptor serine/threonine kinases. Kinase
phosphorylation leads to activation of Smad2 and Smad3, which complex with Smad4 and together bind sites on the enhancer region of the Foxp3 gene (Feng and Derynck 2005; Massague 1998). IL-2R signaling leads to activation of STAT5, which directly binds the Foxp3 promoter, enhancing Foxp3 expression (Burchill et al. 2007).

Foxo1 plays an important role in Treg cell differentiation and function and has been found to bind the Foxp3 locus, controlling Foxp3 promoter activity (Ouyang et al. 2009; Ouyang et al. 2010; Ouyang et al. 2012). Foxo1-deficient T cells, in the presence of TGF-β are misdirected to a Th1 phenotype (Kerdiles et al. 2010). Loss of Foxo1 leads to the inability of TGF-β to suppress T-bet, the Th1 master transcriptional regulator. In addition, Foxo1 directly binds the ifng locus, suppressing IFN-γ expression in Tregs (Ouyang et al. 2012). This indicates an important role of Foxo1 in maintaining stability of the Treg lineage. Foxo1 also regulates cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a co-receptor critical for Treg immunosuppressive function, directly binding upstream of the cttl4 transcriptional start site (Friedline et al. 2009).

Ikaros is a transcription factor that has been shown to regulate T helper cell fates. Ikaros is required for Th2 differentiation, binding to several defined regulatory sites within the Th2 locus as well as to potential Ifnγ regulatory regions. Ikaros also silences ifnγ gene expression by binding to and repressing transcription of the tbx21 gene, which encodes the Th1 master regulator, T-bet (Quirion et al. 2009). In addition, a lack of Ikaros results in significantly reduced expression of Th17 effector cytokines as well as lineage determining factors including RORγt in IK−/− Th17 differentiation cultures.
(Wong et al. 2013). In this chapter, studies show that a lack of Ikaros also leads to profound defects in development of iTregs.

From studies presented in Chapter 3, we were able to establish that Ikaros is required for normal levels of Foxo1 expression. This led us to hypothesize that Ikaros may also be required for iTreg development. In this chapter, we show that similar to Foxo1-deficient T cells, in the absence of Ikaros, CD4 T cells display decreased levels of Foxp3/GFP expression and Foxp3 mRNA levels, observed as early as 24 hours in culture after inducing iTreg differentiation conditions. Retroviral transduction of a constitutively active mutant form of Foxo1, Foxo1-A3, into IK-/ cells was able to increase iTreg differentiation. Therefore, decreased Foxo1 levels contribute to defects in iTreg differentiation in IK-/CD4 T cells. We also show that in the absence of Ikaros, CD4 T cells grown under iTReg differentiation conditions expressed T-bet and RORγt, indicating differentiation into alternative lineages.

Taken together these findings indicate a requirement of Ikaros for regulation of iTreg differentiation.

### 4.2 IK-/ CD4 T Cells Display Reduced Capacity to Attain iTreg Fate

Experiments presented in this chapter involved the use of Foxp3/GFP mice. These mice have an IRES-GFP reporter gene inserted 3’ of the Foxp3 gene, which does not affect expression of Foxp3 (Bettelli et al. 2006). Foxp3-expressing Treg populations in these mice are therefore marked with GFP. For our studies, the Foxp3 reporter mice were crossed to IK-/ and wild type mice. In order to investigate whether a lack of Ikaros leads
to decreased iTreg differentiation, splenocytes or CD4 T cells purified from the spleens of wild type and IK-/− Foxp3/GFP reporter mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of TGF-β and IL-2. After 72 hours, flow cytometry was performed to identify the GFP+ iTreg cells. In the absence of Ikaros, CD4 T cells were crippled in their ability to attain the iTreg fate, as determined by decreased GFP and Foxp3 expression (Figure 16A, 16B). In addition, lack of Ikaros also resulted in a decreased percentage of peripheral Tregs (pTregs) in vivo, as revealed by reduction in percentages of GFP+ cells in spleens of IK-/− Foxp3/GFP reporter mice, indicating that there is a smaller pool of Foxp3 expressing cells available to be induced in IK-/− Foxp3/GFP reporter mice (Figure 16C).
Figure 16. Decreased iTreg differentiation in the absence of Ikaros.

A) GFP expression levels in iTreg differentiation cultures of wild type and Ikaros IK-/ - splenocytes (top panel) as well as purified (bottom panel) splenic CD4 T cell populations using Foxp3/GFP reporter mice. Cells were grown in culture for 72 hours in the presence of 2 ng/ml TGF-β and 50U/ml IL-2 and were removed from anti-CD3 and anti-CD28 stimulation at 24 hours. Data are representative of five independent experiments for each. B) qRT-PCR results for Foxp3 using cDNA from purified CD4 T cells that were grown in culture under iTreg differentiation conditions for 24 hours. (*p<0.05, N=3). C) Percentages of peripheral Tregs in wild type and IK-/ - spleens. (*p<0.05, N=5).
4.3 Lack of iTreg Development in the Absence of Ikaros Cannot Be Attributed to Defective iTreg Survival, TGF-βR/Smad Expression or Effects of Aberrant Cytokine Secretion in vitro

To more precisely define this defect, we investigated explanations for lack of iTreg development in the absence of Ikaros. First, to ensure that the defect was in iTreg differentiation and not the ability of IK-/- iTregs to survive, cultures were analyzed at earlier time points (24 and 48 hours). In wild type cultures, the percentage of GFP+ iTregs increased over time, whereas the IK-/- cultures contained negligible percentages of GFP+ cells at all-time points (Figure 17). These data indicate that decreased survival of IK-/- CD4 T cells at 72 hours is not causing decreased Foxp3 expression, as levels are reduced in comparison to wild type cells as early as 24 hours in culture.

TGF-β is an important cytokine for iTreg cell lineage commitment and Foxp3 is induced downstream of TGF-β signaling. It could be that TGF-βR or its two nuclear effectors, Smad2 and Smad3, may not be expressed in IK-/- CD4 T cells (Feng and Derynck 2005). This also was not the case since IK-/- CD4 T cells expressed equivalent levels of these genes as their wild type counterparts (Figure 18A).

CD4 T cells from a genetically engineered Ikaros mutant mouse strain with deletion of the exon encoding DNA-binding zinc finger 4 (IkΔF4) also display defects in iTreg differentiation (Heller et al. 2014). This was attributed to inappropriate expression by mutant CD4 T cells of large amounts of IL-21, a Th17 cytokine, which can block Foxp3-mediated iTreg differentiation (Heller et al. 2014). To determine if levels of IL-21 were increased in IK-/- iTreg cultures, purified wild-type and IK-/- CD4 T cells were
grown under iTreg differentiation conditions, followed by isolation of RNA after 24 hours in culture. Levels of expression of IL-21 in iTreg differentiation cultures was compared to that expressed by differentiated wild-type Th17 cells. In contrast to cells in IkΔF4 iTreg cultures, which express levels of IL-21 similar to those seen in Th17 cultures, cells in IK-/- iTreg cultures, express negligible levels (Figure 18B). Therefore, the defect underlying lack of iTreg differentiation in the absence of Ikaros cannot be attributed to elevated IL-21 expression.

Upon activation, IK-/- CD4 T cells express increased levels of IFN-γ compared to wild type CD4 T cells (Quirion et al. 2009; Umetsu and Winandy 2009). This occurs under neutral activating conditions, as well as under Th1 and Th2 polarizing conditions. Therefore, increased IFN-γ in IK-/- iTreg cultures could increase T-bet mediated-Th1 cell differentiation. To test if this were occurring in IK-/- iTreg differentiation cultures, anti-IFN-γ was added to determine if iTreg differentiation could be increased. This was unsuccessful, suggesting that alternative cytokines or factors could be compromising Foxp3 expression in IK-/-CD4 T cell iTreg cultures (Figure 18C).

Taken together these data tell us that defects in iTreg differentiation observed in the absence of Ikaros cannot be attributed to decreased TGF-βR or Smad2/3 expression, decreased survival or increased levels of IL-21 and IFN-γ in IK-/- iTreg cultures.
Figure 17. Decreased Foxp3 expression is observed at both early and late time points of iTreg differentiation.

GFP levels of cells that were PI negative at 24, 48 and 72 hours post-culture under iTreg differentiation conditions. Purified splenic CD4 T cells from wild type and IK-/- Foxp3/GFP reporter mice were grown in the presence of 2.0 ng/ml TGF-β and 50U/ml IL-2 and were removed from anti-CD3 and anti-CD28 stimulation at 24 hours.
Figure 18. Defects in TGF-βR, Smad or increased cytokine expression do not cause decreased iTreg differentiation observed in IK-/- CD4 T cells.

A) qRT-PCR results for TGFβR1, SMAD2 and SMAD3 were obtained using cDNA from freshly isolated wild type (black) and IK-/- (dashed) CD4 T cells. Data are average of two independent experiments. B) Quantitative real-time PCR analysis of IL-21 expression using cDNA from wild type and IK-/- purified CD4 T cells grown in culture under iTreg conditions (TGF-β+ IL-2) Wild type cells grown under Th17 polarization conditions (TGF-β, IL-6 and anti-IFN-γ) served as the positive control. Data is normalized to HPRT and is presented as two individual experiments. C) Purified wild type and IK-/- CD4 T cells from Foxp3/GFP mice were grown in culture for 72h in the presence of TGF-β and IL-2 and anti-IFN-γ, followed by flow cytometric analysis. Data is representative of three experiment.
4.4 Rapamycin Cannot Increase iTreg Differentiation to IK-/ CD4 T Cells, But Increasing Levels of Foxo1 Activity Can

In response to TCR stimulation and PI3K/Akt activation, the mTOR pathway acting both upstream and downstream of Akt, coordinates the metabolic state and nutrient demands of a cell according to its need to proliferate, differentiate or maintain tolerance (Howie et al. 2014; Laplante and Sabatini 2009; Hay 2011; Delgoffe et al. 2011) (Figure 19). Immunological tolerance is maintained by Tregs, which induce enzymes that catabolize essential amino acids (EAA) within effector cell populations as well as dendritic cells and macrophages (Howie et al. 2014). Expression of EAA consuming enzymes can begin by an interaction with CTLA-4 on the surface of antigen specific Tregs, or with specific cytokines present in the microenvironment such as TGF-β, IL-4 and IFN-γ (Howie et al. 2014; Cobbold et al. 2009). Depletion of threonine, arginine and in particular tryptophan causes inhibition of the mammalian target of rapamycin (mTOR) pathway, preventing further proliferation of macrophages, dendritic cells and effector cells (Howie et al. 2014). RAG GTPases, a family of Ras-related GTPase enzymes, interact with mTORC1 representing the mechanism known to be responsible for control of mTORC1 activity based on availability of amino acids (Mcegha et al. 2012; E. Kim et al. 2008). However, there is newer evidence suggesting that the binding and activation of the serine/threonine kinase General control nondepressible 2 (GCN2) to uncharged transfer RNA (tRNA), acts as a molecular switch as detection of amino acid levels by mTOR occurs via tRNA synthase-dependent sensing of intracellular amino acid stores (Mcgaha et al. 2012). mTOR activity occurs via two distinct multiprotein complexes
mTORC1 and mTORC2, which differ in how they are activated. The mTORC1 complex contains the scaffolding protein Raptor, as well as the subunits mLST8, PRAS40 and Deptor (Laplante and Sabatini 2009). It acts as the main nutrient sensing complex that promotes phosphorylation of the translational regulators S6K1 and 4E-BP1 and has a central role in regulating cellular growth and proliferation by modulating metabolism and autophagy (Delgoffe et al. 2011; Howie et al. 2014; Holz and Blenis 2005). mTORC1 contains a proline-rich Akt substrate PRAS40 allowing for mTORC1 activation by PI3K and Akt (Delgoffe et al. 2011; Howie et al. 2014). Various signals regulate the mTORC1 pathway, including insulin, hypoxia, mitochondrial function, and glucose and amino acid availability. Such signals are processed upstream of mTORC1 by the Tuberous Sclerosis Complex (TSC1-TSC2) tumor suppressor. This complex acts as an important negative regulator of mTORC1 through its role as a GTPase activating protein (GAP) for Rheb, a guanosine triphosphate (GTP)-binding protein that potently activates the protein kinase activity of mTORC1 (Sancak et al. 2008; Sarbassov et al. 2005). Loss of either TSC protein causes hyperactivation of mTORC1 signaling even in the absence of other upstream signaling such as PI3K/Akt that normally maintain pathway activity. However under conditions of amino acid starvation, the mTORC1 pathway is shut down in cells lacking either TSC1 or TSC2 (Sancak et al. 2008; Smith et al. 2005).

The second mTOR signaling complex, mTORC2, consists of the scaffolding protein Rictor and the subunits mLST8, mS1N1 and Protor (Laplante and Sabatini 2009). Through phosphorylation of the hydrophobic motif of various kinases mTORC2 encourages cell survival via Akt activation, regulates cytoskeletal reorganization by
activating PKCα, and controls ion transport and growth via SGK1 phosphorylation (Laplante and Sabatini 2009). However, the signaling pathways that lead to mTORC2 activation are not well characterized. Growth factors increase mTORC2 kinase activity (Guertin and Sabatini 2007). With growth-factor stimulation, Akt is phosphorylated at the cell membrane through the binding of PIP3 to its pleckstrin homology (PH) domain (Laplante and Sabatini 2009). Interestingly, the mTORC2 component mSIN1 possesses a PH domain at its C-terminus. mSIN1 then promotes the translocation of mTORC2 to the membrane and the phosphorylation of Akt at Ser473 (Laplante and Sabatini 2009). More recent studies however have revealed that PIP3 can directly stimulate the kinase activity of mTORC2 (Gan et al. 2011).

Much of the knowledge about mTOR function comes from the use of the pharmacological inhibitor, rapamycin (Howie et al. 2014; Loewith et al. 2002; Sabatini et al. 1994). mTOR-deficient T cells preferentially differentiate down a Foxp3+ Treg cell pathway (Delgoffe et al. 2009). Rapamycin enhances the generation of inducible Treg cells by shutting down the mTOR pathway. A very low dose of rapamycin of 50 pM inhibits only mTORC1 (Delgoffe et al. 2011) but inhibition of mTORC1 alone is not sufficient for the generation of Treg cells (Delgoffe et al. 2009). Results from studies using a 25nM rapamycin have been representative of a dosage that promotes de novo conversion of Tregs under anti-CD3/CD28 activation conditions (Gao et al. 2007; Sauer et al. 2008). However, it was reported that rapamycin did not rescue the defect in iTreg differentiation observed in Foxo1-deficient CD4 T cells (Kerdiles et al. 2010). To determine if Ikaros is required for Akt/Foxo1 dependent iTreg differentiation, 25nM
rapamycin was added to IK−/− Foxp3/GFP iTreg differentiation cultures. In two independent experiments, rapamycin robustly increased iTreg differentiation in wild type Foxp3/GFP cultures. However, it was unable to do so in IK−/− iTreg cultures (Figure 19). The inability of rapamycin to increase iTreg differentiation suggests that levels of Foxo1 are negligible in IK−/− T cells. However, it could also be that Foxo1 levels are increased with the aid of rapamycin, but a lack of Ikaros still prevents recovery in iTreg differentiation. Therefore, we wanted to directly test if increasing Foxo1 activity by retroviral transduction could increase iTreg differentiation in the absence of Ikaros.

Induction of TCR and cytokine signaling pathways required for iTreg differentiation results in phosphorylation of Foxo1, which leads to its nuclear export and functional inactivation (Van Der Heide, Hoekman, and Smidt 2004). Therefore, to drive nuclear localization of Foxo1 in the presence of these differentiation signals, a constitutively active form of Foxo1, with the three Akt phosphorylation sites mutated to alanine, was used (designated Foxo1-A3) for these experiments (Yusuf et al. 2004). Transduction with Foxo1-A3 was able to increase iTreg differentiation approximately 2-fold in IK−/− cultures, demonstrating that lack of Foxo1 contributes to abrogation of iTreg differentiation in the absence of Ikaros (Figure 20).
In T cells, mammalian target of rapamycin (mTOR) can be activated by multiple signals, including the conventional T cell receptor antigenic stimulation, growth factors and nutrients. The tuberous sclerosis 1 (TSC1)–TSC2 complex upstream of mTORC1 integrates signals such as hypoxia and mitochondrial function. Upon antigen stimulation, TSC is inactivated by T cell receptor (TCR) signals to mediate mTORC1 activation by shutting down RAS homologue enriched in brain (RHEB), but TSC function is maintained in naïve T cells to keep mTOR complex 1 (mTORC1) in check. Amino acids activate mTORC1 via the RAG family of small GTPases. mTORC1 functions to promote translation initiation and protein synthesis by directly phosphorylating the substrates S6 kinases (S6Ks) and eIF4E-binding proteins (4E-BPs). Additional mTORC1 targets include the regulatory proteins in cell signaling, metabolism and autophagy. mTORC2 is
important for full activation of AKT by inducing Ser473 phosphorylation (thus AKT can be both upstream of mTORC1 and downstream of mTORC2) and for phosphorylation of various protein kinases important in regulation of cell survival (Akt), cytoskeletal organization (PKC-α) and cell growth and transport (SGK1).
Figure 20. Rapamycin treatment of IK-/- CD4 T cell cultures does not restore iTreg differentiation.

GFP levels for wild type and IK-/- splenocytes from Foxp3/GFP reporter mice grown under iTreg differentiation conditions with or without 25nM Rapamycin.

Results are representative of two independent experiments.
IK/-/- CD4 T Cells Attain Alternative Fates Under iTreg Differentiation Conditions

IK/-/- CD4 T cells show enhanced differentiation to the Th1 phenotype. Even under strong Th2 polarizing conditions, they skew to the Th1 fate (Quirion et al. 2009). Similarly, cells developing in Foxo1-deficient iTreg cultures appear to be misdirected in their differentiation towards the Th1 lineage in that they express T-bet and IFN-γ (Kerdiles et al. 2010). This led us to ask if cells in IK/-/- iTreg cultures also are aberrantly skewing towards the Th1 effector cell phenotype. iTreg differentiation cultures were set up with freshly isolated IK/-/- and wild type CD4 T cells. Alongside this experiment, wild type CD4 T cells were plated under polarizing conditions to induce Th1 (IL-12, anti-IL-4) and Th17 (TGF-β, IL-6, anti-IFN-γ, anti-IL-4) phenotypes. cDNA made from these wild type differentiation cultures were used as controls to measure the levels of expression of the genes encoding the master regulators of the iTreg, Th1 and Th17 fates. Results from qRT-PCR analyses revealed that cells in IK/-/- iTreg differentiation cultures express high levels of Tbx21 and Rorc, genes encoding T-bet and RORγt, respectively (Figure 21). Therefore, in the absence of Ikaros, CD4 T cells are unable to commit to an iTreg phenotype, but instead express master regulators of the Th1 and/or Th17 lineages, even under strong Treg polarizing conditions.
Figure 21. Re-introducing a constitutively active mutant form of Foxo1 increases iTreg differentiation.

Purified CD4 T cells from spleens of IK-/- and wild type mice were plated under iTreg differentiation conditions and transduced with retroviruses prepared from the MSCV Thy1.1 construct (EV) or the MSCV Foxo1A3 Thy1.1 construct (Foxo1-A3) 24 hours later. Data shown are intracellular Foxp3 staining in successfully transduced Thy1.1+ cells 4 days post-transduction. Results are representative of two independent experiments.
Figure 22. IK−/− CD4 T cells take on alternative phenotypes under iTreg differentiation conditions.

qRT-PCR analyses performed using cDNA prepared from purified wild type (24h WT) and IK−/− (24h IK−/−) CD4 splenic T cells activated for 24 hours under iTreg differentiation conditions. Th0, Th1 and Th17 cultures were comprised of purified wild type CD4 splenic T cells grown in respective subset polarizing conditions (Th0: anti-CD3/anti-CD28 stimulation only, Th1: IL-12 and anti-IL-4, Th17: TGF-β, IL-6, anti-IFN-γ and anti-IL-4) for 5 days.
4.6 Summary and Conclusions

Studies from Chapter 3 established a role for Ikaros in regulation of CD62L and IL-7Rα expression, ensuring proper homing and survival of a naïve CD4 T cell. It was also revealed that Ikaros regulates these genes by increasing levels of Foxo1 expression, as CD62L and IL-7Rα are both direct targets of Foxo1. Foxo1 is also required for development of Tregs. Since a role for Ikaros in Foxo1 regulation has been determined, we investigated if Ikaros may also play a role in Foxo1-driven programs of iTreg differentiation.

It is known that Ikaros is required for differentiation of T helper cell subsets since in its absence, T cells fail to become Th2 cells and default to the Th1 phenotype. In this chapter we have also identified Ikaros as an important regulator of iTreg differentiation. In the absence of Ikaros, there is almost a complete inability of CD4 T cells to differentiate into iTregs. Defective differentiation was not a result of decreased expression of TGF-βR or effectors Smad2 and Smad3.

Although CD4 T cells from another Ikaros mutant mouse strain IkΔF4 also display defects in iTreg differentiation, the high amounts of IL-21 secreted by these cells that were linked to their defective differentiation did not explain defects observed in IK-/- cultures. The IkΔF4 mice have a deletion in exon 4 of the Ikaros gene and express mutant Ikaros protein. This could result in different mechanisms underlying the defective iTreg development than observed in IK-/- mice, which have no Ikaros protein. This might even include dominant negative effects by this DNA binding deficient protein. Also of note,
direct regulation of IL-21 by Ikaros was not demonstrated in those studies.

Under activating conditions, IK-/- CD4 T cells have an increased propensity to become Th1 cells. This is observed even under Th2 differentiating conditions. To investigate if this were occurring in IK-/- iTreg cultures, we added anti-IFN-γ prior to activation and initiation of differentiation. This did not increase iTreg differentiation in IK-/- CD4 T cells.

After having tested for these possible causes, we wanted to investigate if reduced Foxo1 in IK-/- CD4 T cells is causing decreased iTreg differentiation. Rapamycin, a drug shown to increase iTreg differentiation by boosting levels of Foxo1, could not increase levels of Foxp3 expression in IK-/- CD4 iTreg cultures. Similar to what was observed in cell stress experiments presented in Chapter 3, these results suggest that Foxo1 levels are so severely reduced in the absence of Ikaros that encouraging Foxo1 retention in the nucleus is not enough to rescue iTreg differentiation. Retroviral transduction of a constitutively active form of Foxo1, Foxo1-A3, was performed in an attempt to overcome decreased levels of Foxo1 in the absence of Ikaros. Foxo1-A3 did increase iTreg differentiation in IK-/- cultures, although not to the levels of wild-type. These results demonstrate that a lack of Foxo1 in the absence of Ikaros is definitely a contributing factor leading to abrogation of iTreg development. Yet it also suggests that there are additional roles of Ikaros that have yet to be determined.

Having seen that IK-/- CD4 T cells have a decreased ability to become iTregs, it could be that they are differentiating into alternative T helper phenotypes as occurs in iTreg differentiation cultures performed with Foxo1 deficient CD4 T cells. Indeed, IK-/-
cells grown under iTreg differentiation conditions expressed higher levels of T-bet and RORγt, master regulators of the Th1 and Th17 cell fate. Taken together, these data suggest that in the absence of Ikaros, lack of iTreg differentiation is accompanied by the inappropriate expression of factors that potentiate development of effector phenotypes, and Ikaros may therefore also play a role in stabilization of the iTreg phenotype.
CHAPTER 5. DISCUSSION

Experiments presented in this dissertation have contributed to defining a novel Ikaros/Foxo1 axis involved in regulation of naïve CD4 T cell maintenance. We have demonstrated a lack of Foxo1 and Foxo1 target gene expression in the absence of Ikaros. Transduction experiments led to the understanding of a regulatory circuit, placing Ikaros upstream of Foxo1, in mediation of the Foxo1 gene itself as well as its target genes important to naïve T cell survival, homing and iTreg differentiation. Gaining further understanding of this circuit could lead to the potential discovery of therapeutic targets.

5.1 Additional Implications for Ikaros and Foxo1 Regulation of CD62L and IL-7Rα

In Chapter 3, we described a role for Ikaros in regulation of naïve CD4 T cell survival and homing. IK−/− CD4 T cells when adoptively transferred into wild type mice, displayed defective homing to lymph nodes. In addition, they show decreased survival in response to IL-7. IL-7 is also important for homeostatic proliferation of naïve T cells, which allows expansion in the absence of activation and thereby preserves the naïve phenotype (Takada and Jameson 2009). It could be that the decreased ability of IK−/− T cells to respond to IL-7 may contribute to the decreased peripheral T cell compartment observed in the spleens of IK−/− mice.

The ability to respond to IL-7 is also key to maintenance of memory T cells. Foxo1 has been associated with generation and maintenance of CD8 memory T cells (Tejera et al. 2013; M. Kim et al. 2013). Foxo1-deficiency leads to decreased ability of
memory cell formation and survival. This is similar to what is observed in naïve IK-/- CD4 T cells, where we observed reduced CD62L and Il-7Rα expression. It could therefore be that a lack of Ikaros contributes to generation and maintenance of memory CD4 T cells. In support of this, Ikaros restricts autocrine IL-2 production in mature CD4+ T cells (Thomas et al. 2007; Bandyopadhyay et al. 2007) and high levels of IL-2 have been found to interfere with memory cell formation (O’Brien et al. 2014). Therefore, both Foxo1 and Ikaros indicate potential regulatory roles in memory cell formation and maintenance, however mechanisms of regulation are of interest for future research.

5.2 Addressing caveats of the IK-/- mouse model

Numerous developmental defects in CD4 T cells from IK-/- mice were described in Chapter 1. The Ikaros knockout mutation results in decreased hematopoietic stem cell activity where reduced numbers of the earliest multipotent and lineage-restricted precursor populations are observed (Nichogiannopoulu et. al 1999). A lack of Ikaros also compromises processes of positive selection and negative selection, where CD4 lineage development is preferred over CD8 (Urban and Winandy 2004). CD4 T cells lacking Ikaros are also more responsive to less TCR signal, causing them to be more proliferative, also pre-disposing mice to become leukemic (Georgopoulos et al. 1994; Avitahl, 1999). These defects therefore raise important questions regarding the generation of an abnormal IK-/- CD4 T cell population and whether or not results obtained from studies utilizing this model are conclusive. Experiments were therefore designed to address such caveats.
Firstly, mice were euthanized and spleens were harvested at 5 weeks, a critical age preceding the onset of leukemia. In Chapter 1 I discussed experiments that were performed to discount the possibility that IK-/- CD4 T cells expressed decreased levels of CD62L and IL-7Rα because they are abnormally activated or possess a memory phenotype. However, there could still be other potential developmental defects affecting expression levels of these cell surface proteins. To address this, I tested if knocking down expression of either Ikaros or Foxo1 would result in similar defects in wild type cells. Retroviral transductions were performed using a non-DNA binding Ikaros isoform, IK-7 and a Foxo1 shRNA, Foxo1-A1. Wild type cells transduced with IK-7 had reduced cell surface expression of CD62L and IL-7Rα to nearly the same levels as Foxo1 shRNA (Figure 12). These experiments confirmed that defects in expression of Foxo1 targets is in the absence of Ikaros is likely not due to an abnormal cell population. In addition, re-introducing Ikaros into CD4 T cells resulted in increased Foxo1 expression levels in both wild-type and IK-/- populations (Figure 15A) indicating that the ability of IK-/- CD4 T cells to upregulate Foxo1 and its target genes is not compromised in either cell population.

Although the Ikaros null model has exhibited several phenotypic defects profiled in previous studies, experiments for my studies were designed to negate effects of the mutation on proper expression of critical genes regulating CD4 T cell survival, homing and differentiation.
5.3 Further Insight into the Mechanism of Ikaros Regulation of Foxo1 Targets

Findings from retroviral transduction experiments that involved interference with Ikaros or Foxo1 activity via Ik-7 or Foxo1 shRNA, respectively, in wild-type CD4 T cells demonstrated that both Ikaros and Foxo1 are required to maintain high levels of CD62L and IL-7Rα expression. Re-introducing either Foxo1 or Ikaros into IK-/- CD4 T cells highlighted the differential regulation of either Foxo1 target (Figure 13). Transduction of Ikaros into IK-/- CD4 T cells, shown to increase Foxo1 expression, was able to up-regulate IL-7Rα but could not increase CD62L expression. Whereas re-introducing Foxo1 significantly increased CD62L levels, completely bypassing effects of a lack of Ikaros. It therefore appears that Ikaros may be compromising Foxo1's ability to directly bind and activate Sell, indicated by a lack of CD62L upregulation with Ikaros transduction. This could be a result of Ikaros over-expression due to transduction, raising levels higher than those expressed in wild-type cells. Elevated levels of Ikaros could result in Ikaros binding to low affinity sites on the Sell locus, sites to which it would not bind at normal expression levels. Other possible mechanisms by which Ikaros family proteins regulate their targets include competition with transcriptional activators, and recruitment of chromatin modifiers. It could be that increased Ikaros outcompetes binding of co-activators involved in Foxo1 regulation of Sell. Ikaros is known to recruit chromatin remodeling complexes in support of its role in transcriptional activation and repression. It could be that Ikaros interacts with chromatin modifiers, such as the Mi2 histone deacetylase, leading to recruitment of chromatin remodeling complexes to Ikaros binding sites (Merkenschlager 2010; Kim et al. 1999; Bandyopadhyay et al. 2007; Naito...
et al. 2007). This could occur either upstream or downstream of Foxo1 binding along the Sell locus, affecting its ability to activate the Sell gene. Whether any of these mechanisms are involved in Ikaros regulation of the Sell gene need to be further investigated in IK-/-CD4 T cells. Decreased expression of CD62L in wild type cells transduced with Ikaros has not been observed. This could be due to the fact that wild type cells have more stable gene loci not prone to modification based on Ikaros/Foxo1 levels.

5.4 Transcriptional Control of Foxo1

Little is known about how Foxo1 is regulated at the transcriptional level in T cells. To our knowledge, STAT3 is the only described direct activator of the Foxo1 gene in T cells identified to date (Oh et al. 2011; Oh et al. 2012). However, it was identified as an activator of Foxo1 gene transcription in response to IL-6. STAT3 could therefore not contribute to regulation of Foxo1 in naïve T cells, since it would not be activated.

An array of factors have been identified that regulate Foxo1 expression at the transcriptional level during B cell development. E2A and EBF1 bind to regulatory elements within the Foxo1 locus in the common lymphoid progenitor (CLP) (Y. C. Lin et al. 2010). Also within the CLP, microarray studies have revealed that E2A and HEB are required for normal Foxo1 expression, since decreases in Foxo1 expression are observed in the absence of either gene (Welinder et al. 2011).

Ikaros has also been implicated in regulation of the Foxo1 gene in B cells. Foxo1 expression is decreased in Ikaros deficient pro-B cells (Schwickert et al. 2014) and Ikaros directly binds Foxo1 regulatory elements in pre-B cells (Ferreirós-Vidal et al. 2013),
suggesting direct regulation of Foxo1 by Ikaros. In B cells, Ikaros can also indirectly modulate Foxo1 expression through its role in activating expression of genes within the pre-B cell receptor (pre-BCR) signaling pathway, which targets Foxo1 for degradation.

In our studies we found that lack of Ikaros negatively affects Foxo1 protein and mRNA levels. In addition, with Ikaros transduction, qRT-PCR analyses revealed increased levels of Foxo1 expression, suggesting that Ikaros is an important positive regulator of Foxo1 in T cells. Although chromatin from IK-/- CD4 T cells revealed that in the absence of Ikaros, there is less Foxo1 gene activation, these experiments will have to be repeated with appropriate anti-Ikaros antibodies to confirm direct binding of Ikaros to the Foxo1 gene locus in naïve CD4 T cells.

5.5 Additional Modes of Ikaros Regulation of iTreg Differentiation

In Chapter 4 we identified that a lack of Ikaros causes defects in iTreg differentiation. We proceeded to set up retroviral transductions that involved re-introduction of Ikaros into IK-/- Foxp3/GFP CD4 T cells, however we were unable to see increases in Foxp3 expression. The inability of Ikaros to increase Foxo1 and rescue normal iTreg differentiation potential could be due to the limitations of retroviral transduction. According to the protocol, Ikaros transduction did not occur until 24 hours post-initiation of the differentiation culture, which may be too late to restore Foxo1 activity and iTreg differentiation. In order to bypass effects of activation we attempted to use a lentiviral transduction system. However, the use of a lentivirus posed challenges as we had extremely low viral transduction efficiencies and protocols required maintenance
of cells in culture post transfection for 72 hours, without any stimulation. We therefore returned to using the retroviral system, implementing protocols post transduction to restore a resting state, important in driving nuclear localization of Foxo1, which is required for its function. In addition, we used a mutant form of Foxo1, Foxo1-A3 (Yusuf et al. 2004), that was retained in the nucleus post T cell activation, required of retroviral transduction procedures. Retroviral transduction of Foxo1-A3 increased iTreg differentiation potential of IK-/- CD4 T cells about two –fold, but was not rescued to wild type levels.

A lack of Foxo1 contributes to the decrease in iTreg differentiation in the absence of Ikaros, however there are other defects at play suggesting that Ikaros may be regulating other factors in support of iTreg differentiation.

5.6 The Role of Ikaros in Repressing Alternative Lineages in Support of iTreg Differentiation

We show in Chapter 4 that a lack of Ikaros results in a profound defect in iTreg differentiation. We attribute this defect to decreased Foxo1 expression. We also observed that in IK-/- iTreg differentiation cultures, cells expressed levels of Tbx21 and Rorc similar to what was observed in wild type Th1 and Th17 differentiation cultures. Lack of Foxo1 has similar consequences. In iTreg differentiation cultures performed with Foxo1-deficient CD4 T cells, it has been reported that TGF-βR cannot repress T-bet expression, thereby resulting in inappropriate IFN-γ secretion (Kerdiles et al. 2010). Therefore, in the absence of Ikaros, CD4 T cells not only are unable to attain the iTreg fate, but gain characteristics of effector lineages instead. Foxo1 deficiency in the absence of Ikaros
leads to decreased Foxp3 expression. Foxp3 in turn silences numerous target genes that encode transcription factors important to differentiation into alternative lineages (Merkenschlager 2010). Taken together, these data suggest that in both Ikaros and Foxo1 work together to induce iTreg differentiation. Decreased iTreg differentiation can be attributed to the inappropriate expression of factors that potentiate development of effector phenotypes.

Mechanisms of Ikaros regulation of Foxp3 expression have yet to be elucidated. We have identified that Ikaros increases Foxo1, and Foxo1 directly binds the Foxp3 promoter enhancing transcription. An important role for Eos, and Ikaros family member, has been associated with Treg suppressive function. Eos interacts directly with Foxp3 and induces chromatin modifications that result in gene silencing in Tregs (Pan et al. 2009). Silencing of Eos in Tregs compromises their ability to suppress immune responses, instead programming their partial effector function. Whether or not Ikaros carries out a similar mode of regulation requires further study.

**5.7 Future Directions**

Here, we define for the first time a critical role for Ikaros in the naïve T cell and place it upstream of Foxo1 in regulation of naïve CD4 T cell survival, response to stress, homing to lymph nodes and differentiation of iTregs (Gubbels Bupp et al. 2009). Further research into the expression and function of Ikaros in naive CD4 T cells will provide a stronger understanding of Ikaros’ ability to differentially regulate expression of essential genes and factors. In addition, the use of conditional knockout mouse models will identify Ikaros’ role in T cell development in the periphery, escaping thymic
developmental defects observed in the IK-/- mouse model. Restoration of naïve T cell populations is essential to recovery and maintenance of a robust immune response, preventing onset of inflammation and autoimmunity. Further investigation of the role Ikaros may play in mediation of immunological disorders could involve the use of mouse models exhibiting Th1, Th2, Th17 and iTreg-mediated pathology.

There is still much that is not understood regarding how Ikaros regulates Foxo1 expression in naïve CD4 T cells. ChIP experiments will either confirm or deny direct transcriptional regulation of Foxo1 by Ikaros.
List of Journal Abbreviations

Annu. Rev. Immunol......................................Annual Review of Immunology
Antioxid. Redox. Signal............................. Antioxidants & Redox Signaling
Cell Adhes. Migr........................................... Cell Adhesion & Migration
Clin. Dev. Immunol ..................................... Clinical & Developmental Immunology
Cell Metab........................................................ Cell Metabolism
Cell Physiol.................................................... Cell Physiology
Cell Res.......................................................... Cell Research
Curr. Biol........................................................ Current Biology
Curr. Rheumatol. Rep............................... Current Rheumatology Reports
Crit. Rev. Immunol.......................................... Critical Reviews in Immunology
EMBO J............................................................. The EMBO Journal
Eur. J. Immunol............................................ European Journal of Immunology
FEBS lett..................................................... Federation of European Biochemical Societies letters
Genes Dev........................................................ Genes & Development
Immunol. Rev.................................................. Immunological Reviews
Immunol. Today............................................... Immunology Today
J. Autoimmune.................................................. Journal of Autoimmunity
J. Biol. Chem .................................................. Journal of Biological Chemistry
J. Exp. Med .................................................. The Journal of Experimental Medicine
J. Immunol. .................................................. The Journal of Immunology
J. Immunol Res. .................................................. Journal of Immunology Research
J. Med. Gen .................................................. Journal of Medical Genetics
J. Neurosci. .................................................. The Journal of Neuroscience
J. Vis. Exp .................................................. Journal of Visualized Experiments
Mol. Cell. Biol. .................................................. Molecular and Cellular Biology
Nat. Genet. .................................................. Nature Genetics
Nat. Immunol .................................................. Nature Immunology
Nat. Rev. Immunol .................................................. Nature Reviews Immunology
Semin Immunol. .................................................. Seminars in Immunology
Trends Mol Med. .................................................. Trends in Molecular Medicine
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EDUCATION

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University of Waterloo          September 2004-May 2008
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ACADEMIC RESEARCH

Graduate Laboratory Rotation      November 2010-February 2011
Boston University School of Medicine
Advisor: Joel M. Henderson, M.D.
Project: Actn4 Localization in Mutant Mice Samples Representative of Focal Segmental
Glomerulosclerosis via Immuno Gold Electron Microscopy
Techniques: Electron Microscopy, Immuno Gold staining, Mouse kidney isolation and
histological sectioning

Graduate Laboratory Rotation      September-November 2010
Boston University School of Medicine
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Project: Epitope Identification in Francisella tularensis Lipopolysaccharide
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PROFESSIONAL EXPERIENCE

Technical Assistant- Histological  November 2008-August 2010
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- Initiated cell culture laboratory in the department of Biological Engineering, currently involved in passaging several adenocarcinoma cell lines in preparation for rabies vaccine development using microcarrier perfusion systems
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- Prepared fixations of cell culture slides for various histopathological staining procedures – Hoechst, Giemsa, H&E
- Carried out bi-weekly x-ray irradiation experiments on cancer cell line cultures
- Visited MGH’s surgical pathology unit weekly for the retrieval of colonic tumor biopsies
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Summer Intern  May-September 2006
Children’s Hospital Boston- Harvard Medical School

- Assisted Dr. Judah Folkman’s vascular biology team in angiogenesis research, studying the roles of Angiotensin 1 and 2 in the onset of obesity in mice
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• April 2012: International Graduate Student Immunology Conference at Harvard Medical School– Poster presentation: “Role of Ikaros in Foxo1-Driven Gene Expression in CD4 T Cells”

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