

Role of E-proteins in B Lymphocyte Commitment and Thymocyte Selection

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
Immunology in the Graduate School  
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2009

ABSTRACT

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## Abstract

The E-protein transcription factors E2A and HEB regulate various cell processes during the development of B and T lymphocytes, including cell differentiation, lineage commitment, recombination of immune receptor genes, proliferation, and survival. B cell development is dependent on E2A from the earliest stages whereas T cell development relies on the cooperative efforts of both E2A and HEB. Established work demonstrates that the timing and dosage of E-protein expression is critical for mediating these diverse functions. The goal of this dissertation is to develop and utilize new genetic tools to manipulate the timing and dosage of E2A and HEB expression in order to enhance our understanding of E-protein function. Here we develop two new mouse models to identify novel lineage and stage specific roles of E-proteins during B lineage commitment and thymocyte selection.

First, we have generated an E2A inducible mouse model to allow reversible regulation of E2A function and precise timing of induction at the protein level. This system was created by inserting a tamoxifen responsive region of the estrogen receptor ligand binding domain (ER) at the carboxyl end of the *tcfe2a* gene, encoding E2A, to generate E2AER fusion proteins. To our knowledge, the ER fusion system has not yet been tested from an endogenous locus in live animals. Using the E2A<sup>ER</sup> system, we have demonstrated rapidly induced E2AER activity upon tamoxifen treatment that is capable

of supporting B cell development in an *ex vivo* culture system. In addition to characterizing the kinetics and reversibility of this inducible system, we have utilized tamoxifen treatment of E2A<sup>ER</sup> B cell progenitors to identify potential novel E2A target genes driving B lineage commitment.

Second, we have analyzed E-protein function during the double positive (DP) stage of  $\alpha\beta$  T cell development by using a Cre-loxp conditional deletion system. Here, E-protein dosage was manipulated by removal of both E2A and HEB, and the timing of deletion was controlled by using a CD4Cre transgene. During development, survival through the DP stage and initiation of differentiation to the subsequent single positive (SP) stage for generation mature  $\alpha\beta$  T cells is dependent on the production of a functional  $\alpha\beta$  T cell receptor (TCR). The mechanism that maintains cells at the DP stage prior to expression of a mature TCR remains unclear. In this study, we have shown that E2A and HEB together are required to maintain DP fate and regulate the transition to the SP stage. Loss of E2A and HEB in DP thymocytes was sufficient to trigger DP to SP differentiation, even in the absence of a TCR. Deletion of E2A and HEB allowed cells to bypass the requirement for a TCR-mediated positive selection signal. These findings identify E2A and HEB as key regulators enforcing thymocyte positive selection to ensure maturing T cells express a functional receptor.

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## List of Abbreviations

BCR	B cell receptor
bHLH	basic helix-loop-helix
CLP	common lymphoid progenitor
D	diversity gene segment
DC	dendritic cell
DN	double negative
DP	double positive
EBF1	early B cell factor-1
ER	estrogen receptor ligand binding domain
ES	embryonic stem
ETP	early T lineage progenitor
f	flox, conditional allele
GC	germinal center
HMG	high-mobility group
HSC	hematopoietic stem cell
Id	inhibitor of differentiation/DNA binding
Ig	immunoglobulin
IgH	immunoglobulin heavy chain

Ig $\kappa$	immunoglobulin kappa light chain
Ig $\lambda$	immunoglobulin lambda light chain
IgL	immunoglobulin light chain
IgM	immunoglobulin $\mu$ heavy chain
IP	intraperitoneal
ISP	immature single positive
J	joining gene segment
LMPP	lymphoid-primed multipotent progenitor
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MHC I	MHC class I
MHC II	MHC class II
MPP	multipotent progenitor
mTEC	medullary thymic epithelial cell
NFAT	nuclear factor for activation of T cells
NK	natural killer cell
Rag	recombination activating gene
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean

SP	single positive
TCR	T cell receptor
V	variable gene segment

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# 1. Introduction

## 1.1 Overview of lymphocyte development

Development of B and T lymphocytes is a tightly regulated process, and proper maturation of these cells is vital to antigen-specific immune responses. The lymphoid lineages originate from hematopoietic stem cells (HSCs) in the fetal liver or postnatal bone marrow. Postnatally, B and T cell lineages are derived through a series of developmental stages occurring in the bone marrow and thymus, respectively. Development of these two lineages shares several common features. B cell lymphopoiesis entails lineage and stage-specific gene rearrangement at the immunoglobulin (Ig) receptor heavy chain (IgH) and light chain (IgL) loci to produce a diverse repertoire of functional receptors. Stepwise differentiation and rearrangement of Ig genes must be tightly regulated at the transcriptional level to ensure proper generation of antibody producing B cells. For development of  $\alpha\beta$  T cells, a similar process occurs in developing thymocytes at the T cell receptor (TCR)  $\beta$  and  $\alpha$  loci to produce a functional TCR at the cell surface. Once expressing a mature receptor, developing B and T cells undergo a selection process to ensure survival of only non-self-reactive cells. These surviving self-tolerant lymphocytes will then enter the mature B and T cell pools. While the following two sections on B and T lymphopoiesis will cover development from progenitor to mature stages, emphasis will be on the transcriptional regulation of B lineage specification and commitment and on thymocyte selection and

lineage choice. The research presented in this dissertation focuses on these aspects of lymphocyte development.

## **1.2 B lymphopoiesis**

### **1.2.1 B lineage specification and commitment**

#### **1.2.1.1 Progenitor stages**

A complex transcriptional network drives B cell specification and commitment from HSCs through several intermediate progenitor populations. During B cell lineage instruction, progression through early progenitor stages is characterized by gradual priming for B lineage fate and loss of alternative lineage potential (Luc et al., 2008).

First, self-renewing and pluripotent HSCs, which are the source of all blood cell lineages, differentiate into multipotent progenitors (MPPs). MPPs have lost the ability to self-renew, but retain potential for both lymphoid and myeloid lineages (Morrison et al., 1997). The lymphoid lineage consists of B, T, and natural killer (NK) cells whereas the myeloid lineage includes megakaryocytes, erythrocytes, granulocytes, and macrophages. Within the MPP population, a lymphoid-primed multipotent progenitor (LMPP) population has been identified that retains potential for lymphoid, granulocyte, and macrophage differentiation but has lost megakaryocyte and erythroid potential (Adolfsson et al., 2005; Lai and Kondo, 2006; Yang et al., 2005). Subsets of LMPPs have been shown to display expression of several lymphoid specific genes (Igarashi et al., 2002; Mansson et al., 2007), further suggesting lymphoid priming within this population.

The next step in lymphoid development is suggested to be further differentiation of LMPPs to common lymphoid progenitors (CLPs), which demonstrate B, T, and NK cell potential, but have lost remaining myeloid potential (Adolfsson et al., 2001; Kondo et al., 1997). Finally, CLPs that are specified for a B cell fate will transition through the pre-proB cell stage to the proB cell stage, where they will commit to the B cell lineage. The development of HSCs to committed proB cells is depicted in Figure 1.

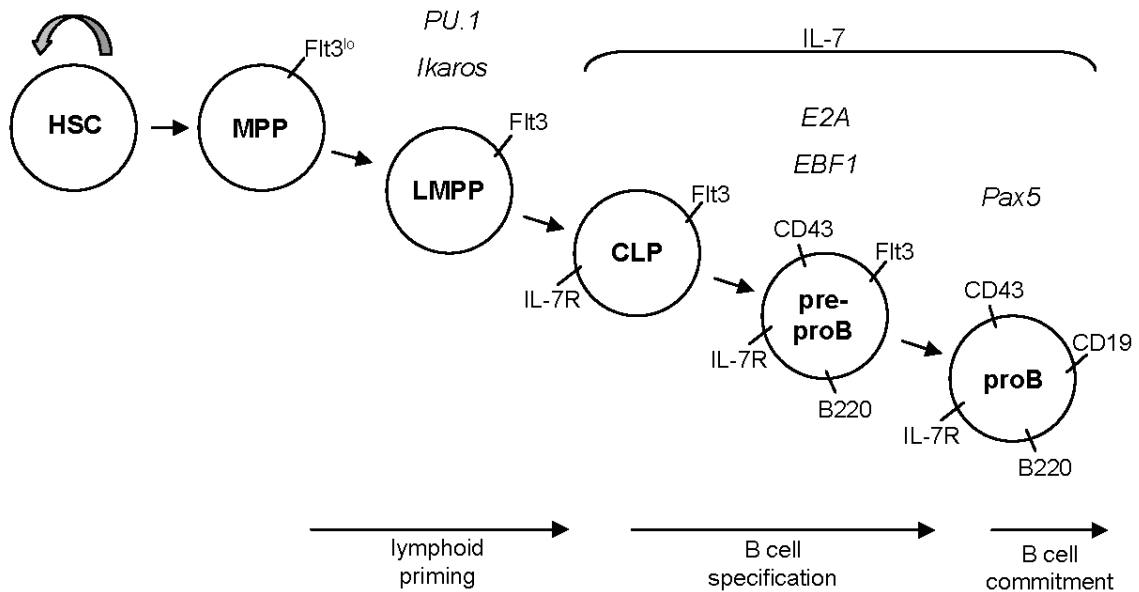


Figure 1: B lineage specification and commitment

B cells originate from HSCs in the bone marrow through several progenitor stages characterized by gradual loss of alternative lineage potential and priming for B lineage development. Self-renewing (circular arrow) HSCs give rise to MPPs. The LMPP population is a lymphoid-primed subset of MPP that is a precursor of the CLP. B cell development is specified as CLPs transition through the pre-proB cell stage to the proB cell stage. Commitment to the B cell lineage occurs at the proB cell stage. Each diagonal arrow indicates further lineage restriction, first with the loss of megakaryocyte/erythrocyte potential at LMPP stage, loss of remaining myeloid lineage potential at CLP stage, and the first step to enter the B cell lineage from a lymphoid progenitor at the pre-proB cell stage. Not until Pax5 expression at the proB cell stage do cells lose all alternative lineage plasticity. Key transcription factors (*italics*) are shown above the stage where development is blocked in the absence of each. While E2A deficiency causes a block at the pre-proB cell stage, E2A has been implicated to function at every stage from HSC through B cell commitment and beyond to mature stages. IL-7 and IL-7R signaling are critical at the CLP stage through proB cell development. Up-regulation of Flt3 expression characterizes the earliest defined lymphoid-primed progenitors. Combinations of B220, CD43, and CD19 expression can be used to identify pre-proB and proB cell stages.

### 1.2.1.2 Transcriptional regulation

Various transcription factors have been identified to contribute to the establishment of the B cell gene expression profile. Key factors include PU.1, Ikaros, E2A, early B cell factor-1 (EBF1), and Pax5. As discussed in more detail below, PU.1 and Ikaros have been implicated in lymphoid priming, E2A and EBF1 are primarily involved in B cell specification, and Pax5 is critical for B lineage commitment. The absence of any of these transcription factors results in a developmental block prior to commitment to the B cell lineage.

The Ets family member PU.1 is one of the earliest known required transcription factors for B cell development. PU.1 deficiency, resulting in severe lethality before or soon after birth, revealed not only a block in B cell development, but also exhibited defects in development of T lymphocytes and myeloid cells (McKercher et al., 1996; Scott et al., 1994). Conditional deletion of PU.1 demonstrated that the block in lymphoid development is prior to the CLP stage (Dakic et al., 2005; Iwasaki et al., 2005). However, deletion of PU.1 in CLPs and B cells post commitment did not significantly impair B cell development, indicating that the essential role for PU.1 is at an earlier stage (Iwasaki et al., 2005; Polli et al., 2005; Ye et al., 2005). There are several other clues that have helped to narrow in on when PU.1 is critical for B cell development. PU.1 expression has been shown to increase from the HSC to the CLP stage (Arinobu et al., 2007; Nutt et al., 2005), and there is a decrease in tyrosine kinase receptor Flt3-expressing progenitors in the

absence of PU.1, along with decreased Flt3 expression levels (DeKoter et al., 2002; Medina et al., 2004). Since LMPPs likely represent the stage of PU.1 up-regulation, are characterized by expression of Flt3 (Adolfsson et al., 2005), and retain potential for the lineages affected by PU.1 deficiency, PU.1 is suspected to function at the LMPP stage. However, the precise role of PU.1 in either the development or function of LMPPs currently remains unclear.

Similar to PU.1, the zinc-finger transcriptional regulator Ikaros has also been shown to play a role during early lymphoid progenitor differentiation. Ikaros has been implicated in activating and repressing gene expression at least in part by influencing chromatin remodeling, given its association with components of the NuRD and SWI-SNF complexes (Kim et al., 1999; O'Neill et al., 2000). Ikaros deficient animals demonstrate severe defects in lymphoid lineages, with a block in B cell development that is suggested to occur prior to the CLP stage (Allman et al., 2003; Georgopoulos et al., 1994; Wang et al., 1996). Recent analysis using an Ikaros reporter mouse indicated that LMPP cells do develop in the absence of Ikaros, but these cells lacked Flt3 expression and as expected, were not able to support B cell differentiation (Yoshida et al., 2006). This work supports a role for Ikaros at the LMPP stage for Flt3 up-regulation and lymphoid priming.

The basic helix-loop-helix (bHLH) transcription factor E2A has also recently been shown to play a role in lymphoid-priming of LMPPs. E2A, a member of the E-protein

family, has been extensively studied for its roles in lymphoid lineage development ever since its identification 20 years ago as an Ig enhancer binding protein (Murre, 2005; Murre et al., 1989a; Murre et al., 1989b). E2A deficiency results in a B cell developmental block at the pre-proB cell stage and a partial block in early T cell development (Bain et al., 1997a; Bain et al., 1994; Zhuang et al., 1994). E2A has been established as a critical regulator of gene expression profiles in both B and T cell progenitors. Now more recently, E2A has been implicated in lymphoid priming by activating a subset of lymphoid-specific genes as early as in the LMPP stage (Dias et al., 2008). This study proposes that E2A regulates lymphoid priming in LMPPs by cooperating with PU.1 and/or Ikaros to induce lymphoid-specific gene expression. Another study demonstrated reduced expression of the recombination activating gene *rag1* at the CLP stage in the absence of E2A (Borghesi et al., 2005). In addition to regulating gene expression, E2A has also been implicated in LMPP and CLP development due to the reduced numbers within these populations in E2A deficient animals (Borghesi et al., 2005; Dias et al., 2008; Semerad et al., 2009). Another recent study even demonstrates reduced numbers of HSCs in the absence of E2A, suggesting that E2A regulates a much broader range of developmental stages than originally anticipated (Semerad et al., 2009).

While these studies demonstrate that E2A functions prior to divergence of the lymphoid lineages, the majority of known functions for E2A during early development identify this transcription factor as a regulator of B lineage specification. One of the key

events for B cell lineage choice is activation of *ebf1*, and E2A is one of the factors that has been shown to induce *ebf1* expression (Kee and Murre, 1998; Roessler et al., 2007; Smith et al., 2002). EBF1 activation has also been shown to be regulated by PU.1 and IL-7R signaling (Kikuchi et al., 2005; Medina et al., 2004; Roessler et al., 2007). EBF1 deficient mice exhibit a block in development at the pre-proB cell stage, similar to that seen in E2A knock-out animals (Lin and Grosschedl, 1995). Together, E2A and EBF1 have been shown to regulate Ig gene recombination and expression of B cell genes including surrogate light chain components (*vpreB* and  $\lambda 5$ ), B cell receptor (BCR) components (*mb-1* and *b29*), and *rag* genes (Gisler and Sigvardsson, 2002; Goebel et al., 2001; Kee and Murre, 1998; O'Riordan and Grosschedl, 1999; Romanow et al., 2000; Sigvardsson et al., 1997).

Another critical B cell specific factor regulated by E2A and EBF1 is the transcription factor Pax5 (O'Riordan and Grosschedl, 1999). While E2A and EBF1 are important for specifying B lineage fate, commitment to the B cell lineage does not occur until expression of their target gene *pax5*. Pax5 deficiency results in a block at the proB cell stage (Urbanek et al., 1994), and Pax5-deficient proB cells still retain the potential to give rise to alternative lineages, including T and myeloid cells (Mikkola et al., 2002; Nutt et al., 1999). E2A, EBF1, and Pax5 appear to act in a hierarchical manner to initiate the B cell lineage gene expression profile. A recent study identifying two promoter regions in the *ebf1* locus and their respective binding factors has reinforced the importance of these

three genes for establishment of the B cell lineage (Roessler et al., 2007). As mentioned previously, E2A has been shown to activate EBF1 expression. E2A, along with STAT5, activates the distal *ebf1* promoter. An auto-regulatory loop is then created as EBF1 is also able to regulate this distal promoter. Pax5 expression is then induced and reinforces EBF1 expression by activating a stronger proximal *ebf1* promoter. This study also demonstrates the ability of PU.1 to activate the proximal promoter. EBF1 regulation is just one example demonstrating the complexity and coordination of B cell transcriptional regulation.

Several other B lineage genes have been identified to be regulated by the coordinated efforts of these transcription factors, including  $\lambda 5$  and *mb-1*.  $\lambda 5$ , a surrogate light chain component, and *mb-1*, the  $Ig\alpha$  signaling unit of the BCR, are both B cell specific factors activated and expressed early during B cell development (Kudo et al., 1987; Travis et al., 1991). Activation of  $\lambda 5$  expression has been shown to be regulated in a synergistic manner by E2A and EBF1 (Sigvardsson, 2000; Sigvardsson et al., 1997). E2A and EBF1 are suggested to form a heterodimeric complex, interacting with both E2A and EBF1 DNA-binding sites in the  $\lambda 5$  promoter (Sigvardsson, 2000). Ikaros has also been implicated in the regulation of  $\lambda 5$  expression. From  $\lambda 5$  promoter studies, Ikaros was suggested to silence expression of  $\lambda 5$  in mature B cells by blocking an EBF1 DNA-binding site (Sabbattini et al., 2001). Similar to  $\lambda 5$ , *mb-1* has been shown to be induced by E2A and EBF1 (Sigvardsson et al., 2002). Following activation, Pax5 is then

required for optimal *mb-1* expression. E2A, EBF1, and Pax5 are proposed to form complexes on the *mb-1* promoter in a stage specific manner that may help stabilize DNA binding or recruit other co-factors. An additional study was able to shed light on the molecular mechanisms regulating *mb-1* expression by demonstrating the ability of E2A and EBF1 to alter the chromatin structure of the *mb-1* locus (Maier et al., 2004). By inducing DNA demethylation and chromatin remodeling, E2A and EBF1 are suggested to allow Pax5 accessibility to the *mb-1* promoter.

### **1.2.1.3 Extracellular signals**

In addition to transcriptional regulation, signals provided by extracellular factors are critical for the development of B cells. For example, the cytokine IL-7 and its receptor, IL-7R, have been shown to be important for B cell development from the CLP stage through proB cell development (Dias et al., 2005; Miller et al., 2002). IL-7 was originally described for its regulation of proB cell proliferation and plays several roles in lymphocyte development (Namen et al., 1988; Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Both IL-7 deficient and IL-7R deficient animals demonstrate a block in development at the pre-proB cell stage (Dias et al., 2005; Kikuchi et al., 2005). While CLP and pre-proB cell numbers are not greatly affected in the absence of IL-7R signaling, B lineage potential is greatly impaired in these cells (Dias et al., 2005; Kikuchi et al., 2008; Kikuchi et al., 2005; Miller et al., 2002). PU.1 activates expression of IL-7R in lymphoid progenitors (DeKoter et al., 2002), and IL-7R signaling is then critical for proper up-

regulation of EBF1 expression in pre-proB cells (Dias et al., 2005; Kikuchi et al., 2005).

The most critical function of IL-7R signaling in B lineage development appears to be activation of EBF1 since enforced EBF1 expression can rescue B cells from IL-7 or IL-7R deficient HSCs or lymphoid progenitors (Dias et al., 2005; Kikuchi et al., 2005).

Regulation of EBF1 by IL-7R signaling is mediated by activation of the downstream signaling molecule STAT5 (Kikuchi et al., 2005). As mentioned earlier, STAT5 has been shown to regulate the distal *ebf1* promoter (Roessler et al., 2007).

IL-7R signaling has been shown to work in coordination with another set of factors, Flt3 ligand and its receptor Flt3, to provide important signals for B cell development. As mentioned above, Flt3 expression is used to characterize the lymphoid-primed progenitor stage, LMPP (Adolfsson et al., 2005). In addition to suggested roles in lymphoid priming, Flt3 expression remains important through pre-proB cell development (Sitnicka et al., 2003; Sitnicka et al., 2002; Sitnicka et al., 2007). Flt3 deficient mice exhibit reduced numbers of pre-proB and proB populations, whereas later developmental stages appear relatively normal (Mackarechtschian et al., 1995). Flt3 ligand deficient mice also demonstrate defects in early B cell developmental stages (Sitnicka et al., 2002). Deficiency of both IL-7R and Flt3 signaling results in a complete block of B cell development, more severe than that observed with each knock-out alone (Sitnicka et al., 2003). IL-7 and Flt3 ligand have been shown to act synergistically to

promote B cell development, further suggesting collaboration between these two cytokine receptor signaling pathways (Veiby et al., 1996).

Recent emphasis has been put on identifying the stromal compartments important for providing these and other lineage determining signals for progenitor cells in the bone marrow. Stromal cells are thought to create organized environments, referred to as niches, which direct the development of B cells (Nagasawa, 2006). This is thought to be particularly important for instructing lineage specification of progenitor populations prior to commitment to the B cell lineage. The lack of markers for bone marrow stromal cells has made it difficult to label bone marrow niches, but some progress has been made. For example, pre-proB cells have been suggested to co-localize with CXCL12 (also known as SDF1) expressing cells, whereas proB cells were seen to co-localize with IL-7-expressing cells (Tokoyoda et al., 2004). Identification of additional stromal markers is expected to further characterize the bone marrow niches critical for B cell development.

## **1.2.2 B cell maturation**

### **1.2.2.1 Immunoglobulin gene rearrangement**

Rearrangement of immune receptor genes in B and T cells, also known as V(D)J recombination, occurs through a tightly regulated process that is mediated by the *rag* genes, *rag1* and *rag2* (Bassing et al., 2002). The variable regions of immune receptors are formed by rearrangement of variable (V), diversity (D), and joining (J) gene segments.

There are several notable features of this process: a large and diverse set of receptors is produced, recombination is lineage and stage specific, and allelic exclusion ensures that each cell has a receptor with a single specificity. Diversity is first created by joining combinations of gene segments from a large array to produce the final rearranged product. In addition, the joining of gene segments is often imprecise as a result of the deletion or addition of nucleotides at gene segment junctions. For example, the *deoxynucleotide transferase* gene product TdT creates variation in the junction sequence by template-independent nucleotide addition (Gilfillan et al., 1993; Kallenbach et al., 1992; Komori et al., 1993). Finally, additional diversity is generated when two chains are paired to form a mature receptor. Lineage and stage specific recombination is achieved by several levels of regulation. Rag1/2 expression is restricted mainly to early stages of lymphocyte development, and accessibility of Rag1/2 to individual receptor loci during development further determines which loci will rearrange. Accessibility can be regulated by enhancers and other cis-acting regulatory elements, specific transcription factors that bind these elements, and chromatin modifications, structure, or nuclear localization (Krangel, 2003; Spicuglia et al., 2006). Allelic exclusion is suggested to be achieved by monoallelic activation of recombination at some loci and negative feedback once a successful rearrangement is produced, inhibiting rearrangement on the other allele (Cedar and Bergman, 2008). This process has also been shown to be regulated, at least in part, at the chromatin level.

In B cells, IgH and IgL pair to form Ig proteins. When associated with the signaling competent transmembrane proteins Ig $\alpha$  (mb-1) and Ig $\beta$  (B29) on the cell surface, IgH/IgL is also referred to as the BCR. Rearrangement of IgH and IgL loci is tightly controlled during development so that IgH recombination precedes IgL recombination. The IgH locus is composed of V, D, and J gene segments that rearrange D<sub>H</sub> to J<sub>H</sub> followed by V<sub>H</sub> to DJ<sub>H</sub>. There are two IgL loci, kappa (Ig $\kappa$ ) and lambda (Ig $\lambda$ ), in mice and humans. These loci contain V and J gene segments. In mice, most cells undergo Ig $\kappa$  recombination prior to Ig $\lambda$  recombination (Arakawa et al., 1996). Rearrangement of these loci is not only important for BCR expression and antigen recognition, but is also a direct regulator of B cell development.

#### **1.2.2.2 Developmental checkpoints**

Once cells commit to the B cell lineage at the proB cell stage they progress through several additional developmental stages before maturing into naïve, functional B cells (Hardy and Hayakawa, 2001). This developmental process is dependent on lineage and stage specific recombination of the IgH and IgL genes. IgH rearrangement occurs at the proB cell stage, and a functional rearrangement leads to surface expression of a pre-BCR. The pre-BCR is composed of the IgH  $\mu$  (IgM) chain, Ig $\alpha$ , Ig $\beta$ , and the surrogate light chain components  $\lambda 5$  and VpreB. Pre-BCR expression allows transition to the preB cell stage and results in clonal expansion followed by rearrangement of the Ig $\kappa$  or Ig $\lambda$  light chain genes. The pre-BCR checkpoint ensures a single functional IgH is

produced prior to continuing with development. Cells that fail to produce a functional IgH rearrangement will undergo apoptosis.

In order to traverse the next checkpoint, preB cells must produce a functional IgL rearrangement. The functional IgH and IgL will form a mature BCR in the form of surface IgM and allow the cells to develop to the immature, or transitional, B cell stage. Immature B cells further mature in the bone marrow or the periphery and may be subject to negative selection. Whether self-reactive immature B cells undergo deletion, anergy, or receptor editing to maintain tolerance continues to be a subject of debate (Allman and Pillai, 2008; Edry and Melamed, 2004; Monroe et al., 2003). Immature B cells give rise to several mature B cell compartments, including follicular, marginal zone, and B-1 B cells. However, positive selection of each of these subsets from the immature B cell pool also remains unclear. A current focus in this area is determining if a ligand-dependent positive selection signal is required or if the expression of a surface BCR on its own is sufficient to supply the positive selection signal (Wang and Clarke, 2004).

## ***1.3 $\alpha\beta$ T lymphopoiesis***

### **1.3.1 Initiation of the T cell lineage**

#### **1.3.1.1 Progenitor stages**

The T cell lineage is established in the thymus from an early T lineage progenitor (ETP) stage. These progenitors give rise to mature  $\gamma\delta$  T cells and all subsets of  $\alpha\beta$  T cells, including NKT cells, regulatory T cells, intraepithelial lymphocytes, CD4 helper T

cells, and CD8 cytotoxic T cells. The direct source of ETPs is believed to be a multipotent progenitor subset derived from the bone marrow that travels through the blood to seed the thymus. Studies have suggested an LMPP-like progenitor expressing Flt3 and the chemokine receptor CCR9 to be the ETP precursor (Benz and Bleul, 2005; Heinzl et al., 2007; Lai and Kondo, 2007; Schwarz et al., 2007; Yoshida et al., 2006). However, the direct precursor of the ETP continues to be a topic of debate. Once in the thymus, ETPs give rise to committed T cells through the coordination of carefully regulated transcription factor expression and signals from thymic microenvironments.

T cell development can be broken down into stages based on cell surface expression of CD4 and CD8. The earliest T cell developmental stages are referred to as double negative (DN) stages due to their lack of CD4 and CD8 expression. DN cells can be further divided into DN1-4 stages based on the following expression of CD44 and CD25: CD44<sup>+</sup>CD25<sup>-</sup> (DN1), CD44<sup>+</sup>CD25<sup>+</sup> (DN2), CD44<sup>-</sup>CD25<sup>+</sup> (DN3), and CD44<sup>-</sup>CD25<sup>-</sup> (DN4). A lot of attention has been devoted to purification of the true T cell precursors within the DN1 population. ETPs have been described as the c-Kit<sup>hi</sup> subset within the DN1 gate, and even that population has been shown to be heterogeneous, containing subsets with varying T cell potency (Allman et al., 2003; Porritt et al., 2004). T cell specification occurs during the transition from the ETP to the DN2 stage, which is then followed by T lineage commitment at the DN3 stage. ETPs and DN2 cells have been shown to still retain potential for alternative lineages including NK, dendritic cell (DC),

and macrophage, but upon development to DN3 stage, this plasticity is lost and cells are locked into a T cell fate (Balciunaite et al., 2005; King et al., 2002; Lu et al., 2005; Shen et al., 2003; Wu et al., 1996).

### **1.3.1.2 Transcriptional regulation**

Similar to their function during B cell development, transcription factors are also critical regulators of lineage specification and commitment throughout T cell development. As discussed earlier, PU.1 and Ikaros are critical at an early lymphoid progenitor stage for generation of both B and T lymphocytes (Dakic et al., 2005; Iwasaki et al., 2005; Yoshida et al., 2006). While PU.1 is critical for lymphocyte development, it is also a regulator of myeloid differentiation, and high levels of PU.1 can divert T cell progenitors to the myeloid or DC lineage (Dionne et al., 2005; Lefebvre et al., 2005). Therefore, PU.1 function must be tightly regulated in developing thymocytes until it is down-regulated at the DN3 stage (David-Fung et al., 2006). Notch signaling in the thymus has been shown to be critical for controlling the outcome of PU.1 expression, allowing functions that promote T lineage progression while inhibiting those that promote a myeloid fate (Franco et al., 2006).

Upon entering the thymus, the Notch signaling pathway is a key regulator functioning during the ETP to DN3 transition for induction of the T cell lineage (Maillard et al., 2005). Notch cell surface receptors, including Notch1-4 in mammals, are activated upon interaction with two families of ligands, Delta-like and Jagged. The

receptor-ligand interaction results in the release of an intracellular region of the receptor that is able to translocate to the nucleus and activate the transcription factor CSL. For T cell development, the Notch1-Delta-like ligand interaction appears to be a critical receptor/ligand combination (Lefort et al., 2006; Radtke et al., 1999; Schmitt et al., 2004; Schmitt and Zuniga-Pflucker, 2002). Notch signaling, in combination with other signaling pathways, initiates development of ETP to the DN2 stage and remains critical into the DN3 stage (Rothenberg et al., 2008). A few of the Notch signaling targets important for T cell development include pre-T $\alpha$  and c-Myc (Palomero et al., 2006; Reizis and Leder, 2002; Weng et al., 2006). However, most of the known downstream Notch targets function after initial commitment to the T cell lineage, leaving the targets of Notch that are critical during early T cell specification largely unknown (Maillard et al., 2005; Weerkamp et al., 2006).

The E-protein transcription factors E2A and HEB have also been shown to be critical regulators of early T cell development and directly regulate a subset of T cell specific genes (Murre, 2005). E2A/HEB heterodimers are the primary E-protein dimers functioning in T cells, and single knockouts of either of these factors results in partial blocks during T cell development (Bain et al., 1997a; Barndt et al., 1999; Barndt et al., 2000; Sawada and Littman, 1993). Conditional deletion of both E2A and HEB in developing T cells with an *lck-Cre* transgene, resulting in a block at DN stage, further revealed the strict requirement for E-protein function during development

(Wojciechowski et al., 2007). One of the targets of E2A and HEB is pre-T $\alpha$ , and E-proteins together with Notch signaling have been suggested to act synergistically to induce pre-T $\alpha$  expression along with other T lineage genes (Herblot et al., 2000; Ikawa et al., 2006; Takeuchi et al., 2001). Additional roles of E-proteins during T cell development will be discussed in more detail in a later section.

Many of these transcription factors, for example PU.1 and E2A, function in the development of multiple hematopoietic lineages. However, in addition to receiving signals specific for T cell lineage instruction (ie. Notch), non-lineage specific transcription factors are still major determinants of cell fate. The specific combinations of transcription factors expressed in the presence of these signals, their order and timing of induction, and their dosage are critical for specifying development of one lineage over another. The cooperation of Notch and E-proteins and the regulation of PU.1 by Notch, both mentioned above, demonstrate the combinatorial effects of transcription factor expression. Gata3, also required for initiation of the T cell lineage, provides a good example of the importance of timing and levels of transcription factor expression to specify T cell development over alternative cell fates.

Gata3 has been described to function throughout T cell development and is critical for T helper type 2 differentiation (Ho and Pai, 2007). However, the exact role of Gata3 in early T cell development has been difficult to ascertain. Since Gata3<sup>-/-</sup> mice are embryonic lethal, the role of Gata3 in establishment the T cell lineage was determined by

*in vivo* reconstitution assays demonstrating the inability of Gata3 deficient embryonic stem (ES) cells to give rise to T cells (Hendriks et al., 1999; Ting et al., 1996). These Gata3<sup>-/-</sup> ES cells were able to reconstitute alternative lineages, including B cells, indicating a T cell specific function at an early stage. Additional studies have shown that Gata3 expression is induced downstream of notch signaling (Amsen et al., 2007; Hoflinger et al., 2004), suggesting Gata3 acts early in T lineage specification. However, over-expression of Gata3 in DN thymocytes is unable to rescue T cell development in the absence of Notch signaling (Taghon et al., 2007). In fact, Gata3 over-expression was shown to block T cell development in the presence of a Notch signal and was able to divert cells to a mast cell fate in the absence of Notch. However, mast cells were only generated by over-expression of Gata3 in DN1 and DN2 thymocytes. Gata3 was not able to change the fate of committed DN3 thymocytes. This study indicates the importance of Gata3 dosage and timing of expression during early T cell development. It is likely that Gata3 is required downstream of Notch signaling, however it also remains possible that low levels of Gata3 are required prior to ETP stage for T cell development. Whether Gata3 is critical in early progenitor stages for directly regulating T cell lineage gene expression or for allowing progenitors to seed the thymus remains unclear.

### 1.3.1.3 Cues from the thymic environment

In addition to Notch signaling, several other extracellular signals critical for T lineage induction have been identified. Both IL-7 and stem cell factor (SCF, the ligand for c-Kit) have been shown to be important during the ETP-DN3 transition (Di Santo and Rodewald, 1998; Massa et al., 2006; Peschon et al., 1994; Rodewald et al., 1995; von Freeden-Jeffry et al., 1995). While Notch signaling has been shown to provide critical T lineage instruction, the downstream signals in response to these cytokines, along with Wnt signaling, have been described predominantly to mediate survival and expansion of developing thymocytes (Ciofani and Zuniga-Pflucker, 2007). However, there may still be some downstream events in response to these signals that are relevant to induction of T lineage genes. Thymocytes are thought to receive most extracellular signals from cytokines and ligands expressed by thymic epithelial cells, as with IL-7, SCF, Notch ligands, and Wnt ligands. Several studies suggest that important signals may be derived from cells of hematopoietic origin as well. For example, double positive (DP) thymocytes, subsequent to the DN stage, have been shown to regulate  $\gamma\delta$  T cell development (Pennington et al., 2003; Silva-Santos et al., 2005). DP thymocytes are also suggested, through lymphotoxin signaling, to regulate gene expression in ETP and DN stages (Silva-Santos et al., 2005).

In order to receive the proper developmental signals in the thymus, ETPs must migrate through the thymic architecture as they mature through the DN stages (Petrie,

2003). ETPs enter the thymus from the blood at the cortico-medullary junction and migrate through the cortex, away from the thymic medulla and toward the supcapsular region. This movement and subsequent development is dependent on the chemokine receptor CXCR4 (Ara et al., 2003; Plotkin et al., 2003). Studies suggest that DN thymocytes compete for access to thymic microenvironments, or stromal niches (Lind et al., 2001; Prockop and Petrie, 2004). Migration through these regions and communication between thymocytes and stromal cells is thought to be critical for specifying non-committed T cell progenitors for a T cell fate (Ciofani and Zuniga-Pflucker, 2007; Takahama, 2006).

### **1.3.2 Developmental checkpoints**

$\alpha\beta$  T cell development in the thymus proceeds through a series of stages defined by surface marker expression and TCR recombination events. Thymocytes undergo recombination of the TCR $\beta$ ,  $\delta$ , and  $\gamma$  loci during the DN2 and DN3 stages. Successful rearrangements at both the TCR $\delta$  and  $\gamma$  loci can result in expression of a  $\gamma\delta$  TCR and development to the  $\gamma\delta$  T cell lineage (Kreslavsky et al., 2008). Successful rearrangement of TCR $\beta$ ,  $D\beta$  to  $J\beta$  followed by  $V\beta$  to  $DJ\beta$ , leads to expression of a pre-TCR and commitment to the  $\alpha\beta$  T cell lineage. The pre-TCR, composed of TCR $\beta$  and pre-T $\alpha$ , signals progression through the immature CD8 single positive (ISP) stage to the DP stage, marked by expression of both CD8 and CD4. Expression of a pre-TCR is the first critical checkpoint during  $\alpha\beta$  T cell development, termed  $\beta$ -selection, to ensure that a

functional  $\beta$  chain is produced prior to continuing with development. Cells that fail to functionally rearrange TCR $\beta$  or TCR $\delta$  and  $\gamma$  will not survive. DN3 cells can be divided into DN3a and DN3b populations, based on CD27 expression, to characterize cells prior to and post  $\beta$ -selection, respectively (Taghon et al., 2006).

Upon development to the DP stage, cells have a limited window of development to undergo TCR $\alpha$  rearrangement,  $V_\alpha$  to  $J_\alpha$ , for production of a mature  $\alpha\beta$  TCR (Huesmann et al., 1991). In response to pre-TCR signaling, the transcription factor ROR $\gamma$ t is up-regulated to create this survival window at the DP stage through regulation of the anti-apoptotic factor Bcl-xL (Kurebayashi et al., 2000; Sun et al., 2000; Xi et al., 2006). DP thymocytes able to functionally rearrange TCR $\alpha$  and express a mature  $\alpha\beta$  TCR on the cell surface during this time can then interact with intrathymic ligands in the context of major histocompatibility complex (MHC) molecules presented on thymic stromal cells. TCR-expressing DP thymocytes receive signals from either MHC class II (MHC II) or class I (MHC I) with the help of the co-receptors CD4 and CD8. CD4 and CD8 bind to MHC II and MHC I, respectively, to promote TCR/MHC interactions and enhance downstream TCR signaling. Depending on their TCR specificity, DP cells give rise to either MHC II-restricted CD4 single positive (SP) cells or MHC I-restricted CD8 SP cells. DP cells must receive a positive selection signal to develop to the SP stage. Cells that fail to produce a functional TCR will not pass the TCR checkpoint and will instead die from neglect. In addition, cells expressing an auto-reactive TCR may be

eliminated by negative selection. The transition from the DP to SP stage, involving positive selection, negative selection, and the decision to enter the CD8 versus the CD4 lineage will be discussed in more detail in the following section.

SP cells surviving selection will exit the thymus to establish the peripheral mature T cell compartments. Up-regulation of the transcription factor KLF2 has been shown to be important for thymic egress by inducing expression of the sphingosine-1-phosphate receptor S1P<sub>1</sub> (Carlson et al., 2006). Expression of the ligand S1P in both the blood and lymph is suggested to mediate egress through both routes (Pappu et al., 2007; Weinreich and Hogquist, 2008). Once in the periphery, mature T cells recirculate through peripheral lymphoid organs, ready to respond upon encounter with antigen.

### **1.3.3 Thymocyte selection and lineage choice**

The diverse repertoire of unique receptors created by recombination of the TCR genes is an immensely valuable feature of the adaptive immune system, providing an extensive range of antigen recognition. However, the fairly random generation of these receptors requires a selective pressure to ensure mature T cells are both self-restricted and self-tolerant. The process of positive selection requires each maturing thymocyte to express a TCR capable of detecting ligand in the presence of self-MHC. Negative selection is critical to eliminate many of those cells that are self-reactive. Once a DP thymocyte expresses a mature TCR and receives a positive selection signal for survival, the cell must also make the decision to enter the CD4 or the CD8 lineage. While positive

selection and lineage choice are tightly linked processes, they are believed to be sequential events. This idea, along with the current models and key factors involved will be discussed below.

### **1.3.3.1 Positive selection**

The majority of DP cells will die because their TCRs can not recognize self-MHC, resulting in a failure to receive a positive selection signal (Surh and Sprent, 1994). The survival signal provided by positive selection has been suggested to be mediated at least in part by up-regulation of the anti-apoptotic factor Bcl2 and by IL-7 signaling (Linette et al., 1994; Strasser et al., 1994; Sudo et al., 1993; Yu et al., 2003; Yu et al., 2006).  $\beta$ -catenin has been implicated in positive selection to enhance IL-7 signaling by up-regulating expression of the receptor component IL-7R $\alpha$  and down-regulating the suppressor of cytokine signaling SOCS-1 (Xu et al., 2003; Yu et al., 2007).

As for the downstream pathways activated by positively selecting TCR signals for differentiation, calcineurin and mitogen-activated protein kinase (MAPK) have been shown to be initiated and critical for formation of both CD4 and CD8 lineages (Alberola-Ila et al., 1995; Neilson et al., 2004; Swan et al., 1995). Calcineurin signaling is activated by calcium and results in activation of nuclear factor for activation of T cells (NFAT) transcription factors (Crabtree, 1999). The up-regulation of Bcl2 and the high-mobility group (HMG) transcription factor TOX, suggested to function in lineage choice, is thought to be mediated by activation of NFAT family members downstream of

calcineurin signaling (Aliahmad and Kaye, 2006; Oukka et al., 1998). Several components of the MAPK pathway have been identified to function during positive selection (Alberola-Ila and Hernandez-Hoyos, 2003). A connection between the TCR and downstream MAPK effectors identified ERK as a critical regulator (Delgado et al., 2000). Specifically, a highly conserved motif in the TCR $\alpha$  chain was identified to be critical for the positive selection signal through ERK activation (Backstrom et al., 1998; Werlen et al., 2000). The transcription factors functioning during positive selection downstream of the ERK MAPK pathway will be discussed in Chapter 4.

#### **1.3.3.2 Models for CD4/CD8 lineage choice**

Several models for CD4/CD8 lineage choice have emerged and evolved to explain how SP thymocytes end up with the correct co-receptor to match their TCR MHC restriction (Singer et al., 2008). Original hypotheses could be divided into either a stochastic or instructive model. The stochastic model proposed that DP thymocytes expressing a functional TCR would randomly shut off expression of either CD4 or CD8, and those cells that correctly matched their co-receptors and TCR specificity (CD4 and MHC II-restricted or CD8 and MHC I-restricted) would survive through the SP stage to maturity (Davis et al., 1993). However, this model was not well supported by accumulating data that “mismatched” thymocytes did not die in the thymus (Keefe et al., 1999; Sarafova et al., 2005). In addition, the efficiency of SP cell development in TCR

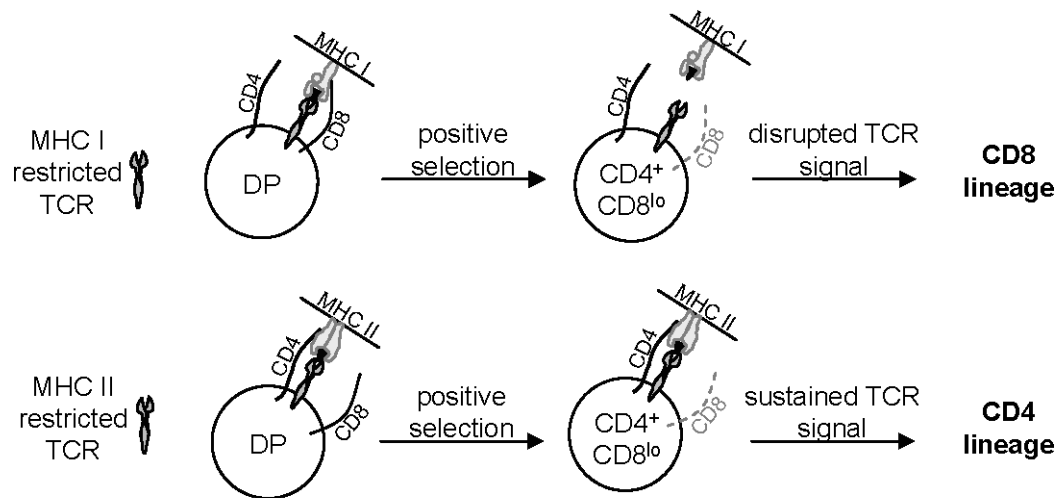
transgenic animals was far greater than the expected 1 out of 2 efficiency for a stochastic system (Itano and Robey, 2000).

As an alternative to the stochastic model, the instructive model suggested that the TCR signal provides instruction for lineage fate (Robey et al., 1991). It was initially suggested that the signal from CD4/MHC II restricted TCR and CD8/MHC I restricted TCR must be distinct, either by quality of the signal or signal strength. Analysis of a transgenic mouse expressing a receptor composed of the CD8 extracellular region with the CD4 transmembrane and cytoplasmic regions demonstrated generation of MHC I restricted CD4 cells (Seong et al., 1992). At the time, interpretation of these results suggested that the signal provided by the intracellular CD4 domain was distinct for CD4 lineage instruction. (However, as will be discussed in detail below, there is another model, the kinetic signaling model, that provides another interpretation of this finding.) Even though there was some support for a difference in signal quality, other studies began to support the idea of a distinct strength of signal from each of the receptor combinations (Hernandez-Hoyos et al., 2000; Itano et al., 1996). Intracellular signaling domains of the CD4 co-receptor were shown to associate with more of the tyrosine kinase lck than was associated with the intracellular domains of the CD8 co-receptor (Ravichandran and Burakoff, 1994; Shaw et al., 1989; Wiest et al., 1993). It was therefore suggested that CD4 provided a strong signal and CD8 provided a weak signal. A role for signal strength in lineage choice was tested by altering the association of lck or

signaling capacity of the intracellular TCR signaling components. While increasing or decreasing signal strength did alter the number of SP cells produced, co-receptors and TCR restriction were still properly matched, demonstrating that the lineage choice outcome was unaffected (Bosselut et al., 2001; Erman et al., 2006; Love et al., 2000). A slight change to the model was suggested; Instead of the strength of signal, perhaps the duration of the signal was different (Brugnera et al., 2000; Yasutomo et al., 2000). This idea led to the development of the currently upheld kinetic signaling model (Brugnera et al., 2000).

The kinetic signaling model is supported by identification of the transitional population DP cells progress through upon receiving a positive selection signal. Once receiving a positive selection signal from ligand presented by either MHC I or MHC II, DP cells down-regulate expression of CD8 at the transcriptional level to become CD4<sup>+</sup>CD8<sup>lo</sup> cells (Bosselut et al., 2003; Brugnera et al., 2000; Lundberg et al., 1995; Suzuki et al., 1995). The resulting loss of CD8 levels on the cell surface will disrupt the TCR signal for an MHC I restricted cell, whereas the signal will be maintained through CD4/TCR for an MHC II restricted cell (Brugnera et al., 2000; Liu and Bosselut, 2004). The duration or cessation of signal is therefore thought to provide lineage instruction for the cell (Figure 2). CD4<sup>+</sup>CD8<sup>lo</sup> cells that experience a disrupted signal will enter the CD8 lineage and must undergo co-receptor reversal to turn off CD4 expression and re-

express CD8.  $CD4^+CD8^{lo}$  cells that experience a maintained signal will enter the CD4 lineage, permanently extinguishing CD8 expression.



**Figure 2: A model for CD4/CD8 lineage choice from positively selected thymocytes**

Once expressing a mature, functional TCR, DP thymocytes can interact with self-ligand (black triangle) presented by MHC on thymic stromal cells to receive a positive selection signal. DP cells express both CD8 and CD4 co-receptors, allowing interaction with either MHC I or MHC II, respectively. The majority of DP cells will fail to receive a positive selection signal due to the failure of their TCR to recognize ligand in the context of self-MHC. The DP cells that are able to receive a positive selection signal will then down-regulate CD8 expression, regardless of their MHC specificity, to enter a  $CD4^+CD8^{lo}$  transitional stage. Upon the resulting reduction of CD8 surface expression (denoted by dotted gray lines), TCR signaling on MHC I restricted cells will be disrupted whereas TCR signaling on MHC II restricted cells will be maintained by the presence of CD4. Cells will interpret this disrupted or persistent signal as instruction for development to the CD8 or CD4 lineage, respectively.

While this kinetic signaling model is the predominantly accepted model, it continues to be experimentally tested. As will be described below, analysis of transcriptional regulation of this event has greatly increased our understanding of CD4/CD8 lineage choice. However, the mechanism that triggers CD8 down-regulation upon positive selection to establish this system for lineage instruction is not entirely understood. Although the intermediate factors remain uncertain, this is likely controlled at the level of CD8 enhancer regulation. Regulation of CD8 enhancers during the DP to SP transition will be further discussed in the following section.

### **1.3.3.3 Transcriptional regulation of lineage choice**

The underlying transcriptional regulation of CD4/CD8 lineage choice has long been an interest and greatly debated topic in the fields of both T cell and developmental biology. The study of transcription factors is essential in order to understand how a surface derived signal can alter the lineage fate of a cell. While there are still many unknowns, several transcription factors have been clearly defined to play a role in CD4/CD8 lineage choice, including Th-POK, Gata3, and Runx factors. Recent study of these factors suggests that the transcriptional network driving lineage fate in positively selected DP cells is far more complex than originally anticipated.

The *zbtb7b* gene, encoding the zinc-finger transcription factor Th-POK (also known as c-Krox), was first found to be critical for the development of the CD4 lineage when it was identified to contain the mutation responsible for the absence of CD4 helper

T cells in a strain referred to as helper deficient (HD) mice (Dave et al., 1998; He et al., 2005). Th-POK has since been characterized as a master regulator of the CD4 lineage (Kappes and He, 2006). Th-POK is required for CD4 lineage development, ectopic expression redirects MHC I restricted cells to the CD4 lineage, and the absence of Th-POK results in MHC II restricted cells entering the CD8 lineage (He et al., 2005; Keefe et al., 1999; Sun et al., 2005). In addition to activating CD4 development, Th-POK also functions to antagonize CD8 lineage development by repressing expression of CD8 and Runx3 (Egawa and Littman, 2008; Jenkinson et al., 2007; Muroi et al., 2008; Wildt et al., 2007). CD4 lineage specific expression of Th-POK is regulated by the combination of enhancer and silencer elements (He et al., 2008; Setoguchi et al., 2008). A persisting TCR signal at the CD4<sup>+</sup>CD8<sup>lo</sup> stage, as is suggested to be required for CD4 development, has been shown to trigger up-regulation of Th-POK expression (He et al., 2008). While the factors responsible for initiation of Th-POK expression are not entirely known, silencing of Th-POK is thought to be relieved by inhibition of Runx proteins (Setoguchi et al., 2008). In addition, recent data suggests that Gata3 functions in part to support the CD4 lineage by activation of Th-POK expression (Wang et al., 2008). Once activated, Th-POK has been shown to amplify its own expression by further antagonizing the Th-POK silencer (Muroi et al., 2008).

Gata3 is another factor up-regulated upon TCR signaling in DP thymocytes (Hernandez-Hoyos et al., 2003). Gata3 was shown to be important for CD4 lineage

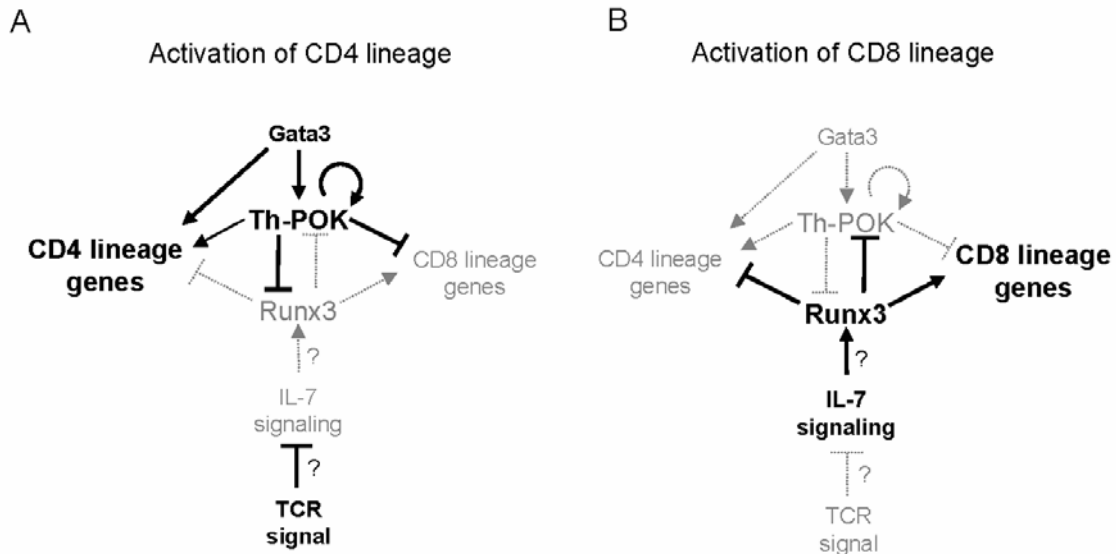
development by studies demonstrating that enforced Gata3 expression inhibited CD8 development and conditional deletion of Gata3 in DP cells resulted in defective CD4 development (Nawijn et al., 2001; Pai et al., 2003). Gata3 has now been placed upstream of ThPOK expression for specification of CD4 lineage choice (Wang et al., 2008). In this study, Gata3 deficient positively selected thymocytes were shown to be blocked prior to initiation of Th-POK expression, and Gata3 was shown to be able to bind regulatory regions of the Th-POK locus. These results suggest that Gata3 functions to activate Th-POK expression for CD4 development. However, because enforced Gata3 expression can not redirect MHC I restricted cells to the CD4 lineage (Hernandez-Hoyos et al., 2003), other currently unknown factors must also be recruited to activate Th-POK. Gata3 not only functions to regulate Th-POK expression, but is also suspected to play additional roles due to the finding that enforced expression of Th-POK in the absence of Gata3 is unable to fully rescue CD4 cells (Wang et al., 2008).

Runx family members, particularly Runx3, are critical transcriptional regulators for CD8 lineage development. Runx factors function to regulate gene expression as heterodimers with the protein Cbfb (Adya et al., 2000). Runx3 expression has been shown to increase as CD4<sup>+</sup>CD8<sup>lo</sup> cells enter the CD8 lineage and is suspected to be critical for co-receptor reversal by repressing CD4 expression and re-inducing expression of CD8 (Sato et al., 2005; Taniuchi et al., 2002). Stage specific expression of CD8 has been shown to be regulated by multiple enhancers (Ellmeier et al., 1997;

Kioussis and Ellmeier, 2002). One enhancer is primarily responsible for expression of CD8 in DP thymocytes and another set regulates expression in CD8 SP cells (Ellmeier et al., 1998). Runx3 has been shown to bind one of the enhancers responsible for CD8 expression in SP thymocytes (Sato et al., 2005). Identification of factors regulating the DP specific CD8 enhancer should shed light on the mechanism regulating the loss of CD8 expression for transition to the CD4<sup>+</sup>CD8<sup>lo</sup> stage. In addition to regulating co-receptor expression, Runx3, along with Runx1, functions to maintain silencing of Th-POK for the development of CD8 cells (Egawa et al., 2007; Setoguchi et al., 2008). Because Runx3 and Th-POK are able to antagonize expression of each other, these two factors are thought to be important for lineage commitment, preventing activation of the alternative lineage. A surprising recent finding was the presence of some CD4<sup>+</sup> T cells in mice deficient for both Th-POK and Cbfb $\beta$ , the Runx binding factor (Egawa and Littman, 2008). This result suggests that other transcription factors are critical for specifying CD4 lineage development prior to Th-POK function (Collins et al., 2009). This finding and the data placing Gata3 upstream of Th-POK (Wang et al., 2008) together suggest that Gata3 may be the factor directing cells to the CD4 lineage in the absence of Th-POK when Runx factors are also absent. A simplified diagram of the transcriptional regulation of CD4/CD8 lineage choice by Th-POK, Gata3, and Runx3 is displayed in Figure 3.

Current data supports the following conclusions: A sustained TCR signal at the CD4<sup>+</sup>CD8<sup>lo</sup> stage initiates Th-POK expression and cells commit to the CD4 lineage; alternatively, if the TCR signal is interrupted, Runx3 drives CD8 lineage development. How then does the cell interpret lineage instruction from the TCR to initiate the correct lineage-specific transcriptional program? While this process is not entirely understood, IL-7 signaling has been suggested to play a critical role in sensing the duration of the TCR signal. Even though positively selected DP cells up-regulate expression of IL-7R $\alpha$ , the TCR signal may be inhibiting IL-7 signal transduction (Brugnera et al., 2000; Sudo et al., 1993). TCR signaling is known to interfere with IL-7 signaling in mature T cells, however it is not known if the same mechanism occurs during the DP to SP transition (Noguchi et al., 1997; Park et al., 2007). If so, the rescue of IL-7 signaling upon disruption of the TCR signal in MHC I restricted CD4<sup>+</sup>CD8<sup>lo</sup> thymocytes may serve as an instructive signal for CD8 lineage development (Singer et al., 2008). Accordingly, IL-7 signaling has been suggested to play a role in co-receptor reversal in part by activating the SP-specific CD8 enhancer (Brugnera et al., 2000; Park et al., 2007; Yu et al., 2003). Due to the similar function displayed by Runx3, IL-7 signaling is hypothesized to function up-stream of Runx3 to instruct development of the CD8 gene expression program (Singer et al., 2008) (Figure 3). However, because IL-7 signaling is critical upon loss of TCR signal for survival (Yu et al., 2003), further investigation is required to identify IL-7 signaling functions specific to CD8 lineage choice.

As mentioned previously, the HMG transcription factor TOX has also been suggested to play a role in lineage choice. However, its precise function is unclear due to some conflicting data. Originally suggested to regulate CD8 lineage over CD4, TOX has more recently been shown to be required for CD4 lineage development (Aliahmad and Kaye, 2008; Aliahmad et al., 2004; Wilkinson et al., 2002). Expression of TOX is rapidly activated after a TCR-mediated positive selection signal in DP thymocytes prior to initiation of both lineages (Wilkinson et al., 2002), suggesting that TOX may function to mediate positive selection instead or in addition to regulating lineage choice.



**Figure 3: Transcriptional regulation of CD4/CD8 lineage choice.**

(A) Activation of the CD4 lineage is dependent on transcription factors Th-POK and Gata3. In addition to activating CD4 lineage genes, Th-POK forms an auto-regulatory loop to enforce CD4 lineage choice and suppresses CD8 lineage genes including Runx3. Gata3 functions up-stream of Th-POK activation and provides some other essential Th-POK independent regulatory event for CD4 lineage development. Gata3 and Th-POK expression are thought to result from the persisting TCR signal for instruction of CD4 lineage choice. This maintained TCR signal may also inhibit IL-7 and other cytokine signaling to prevent CD8 lineage development. (B) Activation of the CD8 lineage is dependent on Runx3. Runx3 also provides dual functions, promoting expression of CD8 lineage genes while repressing CD4 lineage genes including Th-POK. The antagonistic actions of Runx3 and Th-POK are critical to reinforce lineage choice. IL-7 signaling is suggested to be restored upon disruption of TCR signaling in CD4<sup>+</sup>CD8<sup>lo</sup> cells for CD8 lineage instruction and may function up-stream of Runx3 activation. Active factors (**bold**) and repressed factors (**gray**).

#### 1.3.3.4 Negative selection and thymocyte migration

Negative selection is mediated by the proper localization of developing thymocytes for interactions with self-ligands and the resulting apoptotic signal

identifying an auto-reactive TCR. Several chemokine receptors have been identified to play a role in thymocyte migration for proper negative selection. For example, CCR7 is up-regulated in positively selected thymocytes and is critical for subsequent migration from the cortex to medulla (Campbell et al., 1999; Kwan and Killeen, 2004; Misslitz et al., 2004; Suzuki et al., 1999; Ueno et al., 2004). Migration to the medulla is important for establishing tolerance (Palmer, 2003). While SP maturation, survival, and egress from the thymus are not dependent on cortex to medulla migration, negative selection is greatly impaired when this migration step is blocked (Kurobe et al., 2006; Ueno et al., 2004). Mice deficient for CCR7, or its ligands CCL19 and CCL21, demonstrate autoimmune phenotypes including lymphocyte infiltrations and tissue damage in peripheral gland tissues (Kurobe et al., 2006). This phenotype likely results from the lack of contact between developing thymocytes and medullary thymic epithelial cells (mTECs). MTECs express tissue specific antigens to mediate deletion of auto-reactive T cell clones. For example, the transcriptional regulator Aire is responsible for inducing expression of a set of tissue specific antigens in mTECs, and autoimmune disorders result in its absence (Anderson et al., 2005; Kuroda et al., 2005; Liston et al., 2003; Zuklys et al., 2000).

The downstream events driving TCR-mediated apoptosis during negative selection have been shown to be mediated by Bcl2-regulated pathways (Strasser et al., 2008). The BH3-only pro-apoptotic factor Bim was identified to specifically regulate

apoptosis in thymocytes in response to a negative selection signal (Bouillet and Strasser, 2002; Villunger et al., 2004). As a result, Bim deficient animals also develop autoimmunity (Bouillet et al., 1999). Bim activity is thought to be induced downstream of calcium signaling from a strong TCR signal for negative selection (Bunin et al., 2005; Cante-Barrett et al., 2006), but its direct regulators remain undefined. An additional question of interest is how the TCR signal is interpreted as a positive selection/survival signal versus a negative selection/cell death signal. While low affinity and high affinity interactions are known to result in positive and negative selection, respectively (Starr et al., 2003), additional work must be done to determine what specific roles ligand affinity, signal strength and duration, and other contributing signaling pathways play. Differences in these aspects likely alter the levels or localization of intracellular signaling molecules to ultimately direct the cell's selection fate (Daniels et al., 2006; McNeil et al., 2005; Prasad et al., 2009).

## ***1.4 E-protein regulation of lymphocyte development***

### **1.4.1 Basic helix-loop-helix transcription factors**

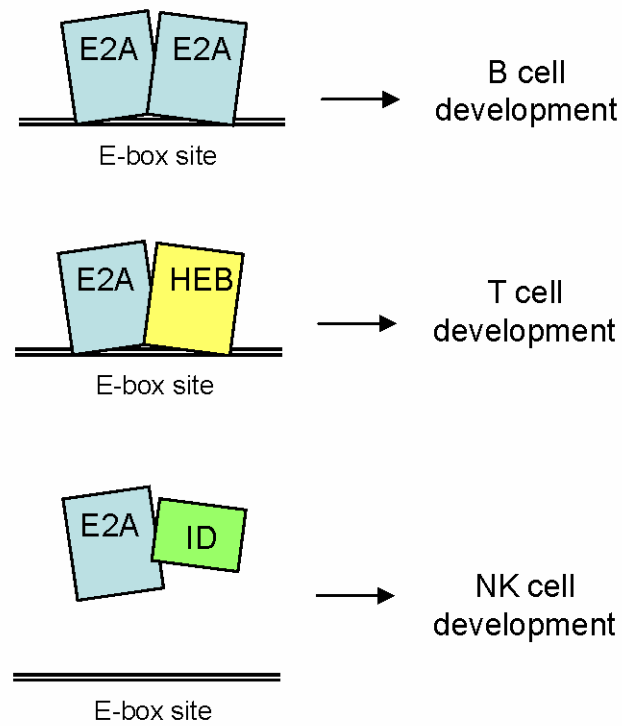
Class I basic helix-loop-helix (bHLH) transcription factors, also known as E-proteins, include three family members in mammals: E2A, HEB, and E2-2. The *tcfe2a* gene, encoding E2A proteins, produces two gene products, E47 and E12, from alternative splicing. E-proteins function as dimers, binding DNA at consensus sites referred to as E-box sites (CANNTG) to regulate gene expression (Murre et al., 1989a).

Dimerization is mediated through the HLH domain whereas DNA binding requires the basic region. Transcriptional regulation is mediated through two activation domains (Aronheim et al., 1993; Bhalla et al., 2008; Massari et al., 1996; Quong et al., 1993). E-proteins can function as homodimers or heterodimers with other HLH factors (Massari and Murre, 2000). In many tissue types, E-proteins form heterodimers with tissue-specific class II bHLH proteins. For example, E-proteins heterodimerize with MyoD and NeuroD to regulate myogenesis and neuronal development, respectively (Lassar et al., 1991; Murre et al., 1989b; Naya et al., 1995). However, E-proteins primarily dimerize with other E-proteins to drive development in lymphocytes.

Another group of HLH factors, referred to as class V, has the ability to negatively regulate class I and class II bHLH factors (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990). This group includes the inhibitor of differentiation (Id) genes, *Id1-4*. Id proteins contain the HLH domain for dimerization, but lack the basic region required for DNA binding. Therefore, Id proteins are able to inhibit E-protein DNA binding activity through competitive dimerization. Id2 and Id3 have been shown to be particularly important for regulating E-protein activity during lymphocyte development (Pan et al., 1999; Rivera et al., 2000; Yokota et al., 1999).

E2A is the founding member of the E-protein family in mammals, first identified by its ability to bind Ig enhancer regions (Church et al., 1985; Ephrussi et al., 1985; Murre et al., 1989b). E2A was quickly suspected to play a broad role in development due to its

homology to *daughterless*, the *Drosophila* gene involved in cell determination and differentiation (Jan and Jan, 1993; Murre et al., 1989b). Following their identification, both E2A and HEB have been intensely studied for roles in lymphocyte development (Murre, 2005). While E-proteins are widely expressed in multiple tissue types, the expression of E2A and HEB during lymphocyte development is tightly regulated (David-Fung et al., 2006; Engel et al., 2001; Herblot et al., 2002; Zhuang et al., 2004). E2A homodimers and E2A/HEB heterodimers are the primary E-protein dimers functioning in B and T lymphocytes, respectively (Barndt et al., 2000; Sawada and Littman, 1993; Zhuang et al., 1996) (Figure 4). Inhibition of E-protein activity by Id proteins is critical for NK cell development (Boos et al., 2007; Yokota et al., 1999). However, concentration here will be on the role of E2A in B cell development and the role of E2A/HEB in T cell development.



**Figure 4: E-protein dimers driving lymphocyte development**

E-proteins function to regulate gene expression by forming dimers to bind DNA sequences referred to as E-box sites. The HLH domain is critical for dimerization and the basic region is required for DNA binding. E2A homodimers are important for the development of the B cell lineage whereas E2A/HEB heterodimers are important regulators of T cell development. Id proteins, lacking the basic region, can dimerize with E-proteins to inhibit DNA binding activity. Inhibition of E-proteins by Id proteins is important for the development of the NK cell lineage.

### **1.4.2 Overview of E2A function during B cell development**

E2A is not only critical for progenitors to enter the B cell lineage, but continues to function by regulating gene expression, V(D)J recombination, proliferation, and survival throughout B cell development and maturation. E2A regulates expression of genes

critical to the B cell lineage including *ebf1*, *pax5*, *vpreB*,  $\lambda 5$ , and *mb-1* (Lazorchak et al., 2005). EBF and Pax5, as discussed previously, cooperate with E2A to establish the B lineage gene expression profile. While E2A is important for initiation of B lineage gene expression, it does not appear to be required for maintenance of some of these factors. Deletion of E2A in preB cell lines does not significantly alter the expression of genes such as *ebf1* and *mb-1* (Greenbaum et al., 2004), indicating that maintenance of these genes is E-protein independent or the presence of low levels of other E-proteins, such as E2-2, may be sufficient to compensate. In addition to activating B lineage genes, E2A has been implicated in repressing alternative lineage genes, for example the erythroid developmental factors *gata1* and *epoR* (Ikawa et al., 2004).

E2A also plays several important roles in the locus activation, recombination, and surface expression of Ig receptors. First, E2A directly binds to sites within the IgH and Ig $\kappa$  enhancers, and ectopic E2A expression in non-B cell lines has been shown to be sufficient to activate expression of *IgH* and *IgL* germline transcripts (Choi et al., 1996; Greenbaum et al., 2004; Greenbaum and Zhuang, 2002; Henthorn et al., 1990; Kee and Murre, 1998; Murre et al., 1991; Romanow et al., 2000; Schlissel et al., 1991). Ectopic E2A expression not only induces transcription, but also results in recombination upon co-expression with Rag1 and Rag2 (Goebel et al., 2001; Romanow et al., 2000; Schlissel et al., 1991). E2A's effect on recombination could be direct, could be due to increased accessibility upon E2A-induced transcription, or could be due to a combination of

functions (Agata et al., 2007; Jones and Zhuang, 2008; Lazorchak et al., 2006a). E2A has also been shown by several studies to regulate expression of the *rag* genes and can directly interact with the *erag* enhancer, critical for Rag expression in B-cells (Hsu et al., 2003; Kee and Murre, 1998; Schlissel et al., 1991). In addition, TdT has been shown to be a direct target of E2A that functions during recombination (Choi et al., 1996; Greenbaum and Zhuang, 2002). Finally, E2A-mediated expression of pre-BCR and BCR components, for example VpreB,  $\lambda 5$ , and mb-1, is critical for assembly of the receptor chains at the cell surface.

During the development of mature B cells, E2A dosage has been suggested to play a role in the determination of follicular versus marginal zone B cell fate. High levels of E2A favor the development of follicular B cells while high levels of Id3, resulting in lower E2A activity, favor marginal zone B cell development (Quong et al., 2004). E2-2 has also been implicated to influence the developmental choice to become a marginal zone or follicular B cell (Wikstrom et al., 2006). In mature resting B cells, E2A expression is relatively low, but is increased upon B cell stimulation (Quong et al., 1999). E2A has been shown to function in activated germinal center B cells, however the mechanism is unclear (Kwon et al., 2008). E2A has been suggested to play a role in class switch recombination and somatic hypermutation by regulating expression of activation-induced cytidine deaminase (AID) (Goldfarb et al., 1996; Hauser et al., 2008; Sayegh et al., 2003). In addition, E2A is thought to be critical for survival of mature B

cells (Lazorchak et al., 2006b). However, many of the mechanisms mediating these E2A functions in peripheral B cells are currently undefined.

### **1.4.3 Overview of E2A and HEB function during T cell development**

As with E2A function in B cells, the roles of E2A and HEB in T cells continue beyond lineage commitment. E-protein regulation of TCR recombination is similar to that observed at the Ig loci. E-box sites are located within TCR regulatory regions, and ectopic expression of E2A and/or HEB in non-lymphoid cell lines can activate  $V\gamma$  and  $V\delta$  germline transcription and recombination in the presence of Rag proteins (Ghosh et al., 2001; Gottschalk and Leiden, 1990; Ho et al., 1989; Takeda et al., 1990). E2A has also recently been shown to regulate transcription and recombination at the TCR $\beta$  locus (Agata et al., 2007; Jia et al., 2008). E-proteins then regulate the pre-TCR checkpoint by inducing expression of pre-T $\alpha$ , but also by preventing further development until a functional TCR $\beta$  is produced (Herblot et al., 2000; Takeuchi et al., 2001; Wojciechowski et al., 2007). E2A and HEB are required to inhibit cell cycle progression of DN3 thymocytes prior to a pre-TCR signal (Engel and Murre, 2004; Wojciechowski et al., 2007).

After the pre-TCR checkpoint, E-proteins are suspected to be involved in development from the ISP to the DP stage due to the accumulation of ISP cells in HEB deficient mice and due to the interaction of E2A and HEB with the CD4 enhancer (Sawada and Littman, 1993; Zhuang et al., 1996). E-proteins have also been suggested to

play a role during positive selection at the TCR checkpoint. As this is a major research interest of this dissertation, the role of E2A and HEB during the DP to SP transition will be further discussed in Chapter 4.

While many E-protein functions have been identified during T cell development, very little is known about the role of E-proteins in mature T cells. Similar to mature B cells, E2A expression is low in resting T cells and up-regulated upon stimulation (Pan et al., 1999). E2A deficient mature T cells demonstrate alterations in proliferation both before and after TCR stimulation, and Id2 has been shown to regulate CD8 effector T cell responses, likely by controlling E-protein levels (Cannarile et al., 2006; Pan et al., 1999). Additional functions for E-proteins in effector lymphocytes are likely to be uncovered with the use of conditional deletion systems in mature cells to separate developmental and effector defects.

## **2. Materials and methods**

### **2.1 Mice**

E47<sup>bm</sup>, Tcfe2a<sup>flox</sup>, E $\alpha^{\Delta}$ , Tcf12<sup>flox</sup>, CD4Cre transgenic, and LAT<sup>-/-</sup> mice have been described previously (Pan et al., 2002; Sleckman et al., 1997; Wojciechowski et al., 2007; Wolfer et al., 2001; Zhang et al., 1999; Zhuang et al., 1998). Rag2<sup>-/-</sup> mice were a gift from Dr. Michael Krangel's lab. Generation of E2A<sup>ER</sup> mice is described below. All research with mice was performed in accordance with relevant guidelines, and protocols were approved by the Duke University Animal Care and Use Committee.

### **2.2 Generation of E2A<sup>ER</sup> mice**

The gene targeting strategy used was a modification of the strategy for generation of E2A<sup>GFP</sup> mice (Zhuang et al., 2004). The tamoxifen-responsive region of the mouse estrogen receptor ligand binding domain containing the G525R mutation (Danielian et al., 1993) was PCR amplified from the MigR1-E47R vector (Zhao et al., 2001) using the primers ERfpA: 5'-CGGATCCACGAAATGAAATGGGTGC-3' and ERrpA: 5'-CCGGCCGCTAGAATTCGATCGTGTGGGGAAGCCCTC-3' to introduce a 5' BamHI site and 3' EcoRI and EagI sites for subsequent cloning steps. The ER fragment was inserted, replacing EGFP, at the BamHI position in frame with E2A. The targeting construct also contained a positive selection marker, a PGKNeo cassette, and a negative selection marker, a PGK driven thymidine kinase (TK) cassette. Mouse ES cells used were derived from a 129/sv strain obtained from Phillippe Soriano's lab in 1995

and then maintained in our own lab. E2A<sup>ER</sup> mice were maintained on a C57BL6 and 129/sv mixed background. Three primers were used for detection of wild-type and mutant alleles, yz164: 5'-AAGAACGAGGCCTTCCGTGTC-3', yz29: 5'-TCGCAGCGCATCGCCTTCTA-3', and bjE2Ar3: 5'-CAAGAGACTAGGATGCCACTG-3'.

### **2.3 RT-PCR**

For E2A, Pax5, Trib2, and TCR $\alpha$  analysis, total RNA was extracted using TRIzol (Invitrogen) and isopropanol precipitation. RNA was treated with RNase free DNase I (Sigma-Aldrich) prior to reverse transcription. For ROR $\gamma$ t, Gfi1, Mad111, KLF2, and Foxo1 analysis, RNA was extracted using RNeasy QIAGEN kit with DNase I step following manufacturer's protocol. Random primed cDNA was made using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed using a Roche LightCycler and Fast-Start DNA master SYBR green kit I (Roche) as per manufacturer's instructions. See Table 1 for RT-PCR primer sequences. Pax5 and GAPDH primers (Lazorchak et al., 2006b), ROR $\gamma$ t primers (Xi et al., 2006).

**Table 1: RT-PCR primer sequences**

<b>Primers: 5'-3'</b>	
GAPDH for	CCT GGA GAA ACC TGC CAA GTA TG
GAPDH rev	AGA GTG GGA GTT GCT GTT GAA GTC
E2A f1	CCA GTC TCA GAG AAT GGC AC
E2A r1	CCT TCG CTG TAT GTC CGG CTA G
Pax5 for	CCG CCA AAG GAT AGT GGA ACT TG
Pax5 rev	CAC AGT GTC ATT GTC ACA GAC TCG C
Trib2 for	CGA GAC TCC GAA CTT GTC GC
Trib2 rev	GCA ATC TGG TAG AAC AGT CG
C $\alpha$ for	CTG CCT GTT CAC CGA CTT TGA C
C $\alpha$ rev	GAT TCG GAG TCC CAT AAC TGA CAG
Gfi1 for	GAT TCC ACC AGA AGT CAG
Gfi1 rev	GGT AGT GTT ACA CAG CTG GTG
Mad1 F1	GCT GAC CGG CTA CCA GAT TG
Mad1 R1	GCT CAG GCA CAG ACC GTG AG
KLF2 F4	CCT ACA CCA AGA GCT CGC ACC
KLF2 R4	GTC GAC CCA GGC TAC ATG
Foxo1 F1	GGA CTG TGA CAT GGA GTC C
Foxo1 R1	GGA CTG CTC CTC AGT TCC TG
ROR $\gamma$ t for	TGT CCT GGG CTA CCC TAC TG
ROR $\gamma$ t rev	GTG CAG GAG TAG GCC ACA TT

## **2.4 Cell staining and flow-cytometry**

Intracellular staining was done by 2% paraformaldehyde fixation followed by permeabilizing in 0.5% saponin. Annexin V staining was done according to the manufacturer's protocol (BD Pharmingen). For culture analysis pre-proB cell sorting, bone marrow was harvested and pooled from 2-3 mice per genotype. For RNA collection from wild-type pre-proB and proB cells, 3 C57BL6 mice were used for 3 replicate samples. Cells positive for lineage markers Mac-1, Gr-1, Ter-119, and CD3

were depleted with Dynal Dynabeads (Invitrogen) according to manufacturer's instructions. Dead cells stained with 7-aminoactinomycin D (7AAD, Molecular Probes) were excluded. FACS analysis was done with a FACSCalibur (BD Biosciences) or FACSVantage SE with DiVa option (BD Biosciences) and FlowJo software (Tree Star). FACSVantage SE with DiVa option was used for cell sorting.

## ***2.5 In vivo BrdU labeling***

Mice were intraperitoneal (IP) injected with 100ul of 10mg/mL BrdU in sterile 1X DPBS for a total of 1mg BrdU/mouse. Analysis was done 4 or 24hrs post-injection with a FITC BrdU Flow Kit as per manufacturer's instructions (BD Pharmingen).

## ***2.6 Tamoxifen preparation***

Tamoxifen (Sigma) was prepared as a 1mM stock (1000x) dissolved in cell culture grade dimethyl sulfoxide (DMSO) and stored at -20°C.

## ***2.7 Abelson transformed preB cell lines***

The E2A<sup>ER</sup> Abelson preB cell line was derived by Abelson Murine Leukemia Virus transformation of bone marrow cells from an E2A<sup>ER</sup> homozygous mouse. Briefly, whole bone marrow was plated on an S17 stromal layer in the presence of 1uM tamoxifen and 10ng/mL IL-7 in 5% FBS RPMI media. This culture was performed prior to transduction to ensure cells are proliferating and are at the optimal target stage for Abelson transformation. Once an expanding B cell population was observed, cells were infected with Abelson virus in the presence of 4ug/mL polybrene. Abelson transformed

cells were then removed from the stromal layer, and tamoxifen and IL-7 were withdrawn. The established E2A<sup>ER</sup> Abelson preB cell line was maintained in 10% FBS RPMI media (also containing 100units/ml penicillin, 100ug/mL streptomycin and 55uM 2-mercaptoethanol) prior to experimental analysis.

## **2.8 Electrophoretic mobility shift assay**

E2A<sup>ER</sup> Abelson preB cells were cultured with or without 1uM tamoxifen as indicated. For withdrawal analysis, tamoxifen-treated cells were washed and re-plated in the absence of tamoxifen for the indicated times. Nuclear extracts were incubated with a <sup>32</sup>P-labeled  $\mu$ E5 oligonucleotide probe, with or without Yae anti-E2A monoclonal antibody (Santa Cruz Biotechnology, sc-416), and resolved on a 5% polyacrylamide gel. Gels were dried and exposed to a phosphor screen for phosphorimager analysis (Amersham Biosciences). Oligos used for  $\mu$ E5 probe: 5'-TCGAAGAACACCTGCAGCAGCT-3' and 5'-TAGAGCTGCTGCAGGTGTTCTT-3'.

## **2.9 In vivo tamoxifen treatment**

Mice were treated with tamoxifen in the drinking water for 27 days. A 68mg/mL tamoxifen in ethanol stock was used to bring the concentration in drinking water to approximately 26ug/mL, resulting in 0.04% ethanol in water. A fresh bottle of tamoxifen water was given every 5 days.

## **2.10 Ex vivo pre-proB culture system**

Sorted pre-proB cells were plated on an S17 stromal layer in 24-well plates at approximately  $1.5 \times 10^4$  cells per well and cultured with 5% FBS RPMI hormone-free media containing 10ng/mL IL-7. Hormone free media consisted of phenol-red free RPMI 1640 supplemented with 5% charcoal/dextran treated FBS (Hyclone), 100units/mL penicillin, 100ug/mL streptomycin, and 55uM 2-mercaptoethanol. Treated wells contained 1uM tamoxifen and untreated controls were given DMSO alone (0.1%). Cells were harvested at time points indicated, and samples were split in half for FACS analysis and RNA extraction.

## **2.11 E2A and HEB deletion efficiency analysis**

DNA was isolated from sorted cells by lysis in a Triton lysis buffer (Tris-EDTA, 0.2% Triton X100, 0.2mg/mL proteinase K) at 55°C for 30min, 95°C for 10min, and then cooled to 4°C. E2A deletion was determined by two independent methods because the difference in product size renders quantification of deletion difficult from a single PCR reaction. First a quantitative PCR method to detect percent deletion is performed using the following primers: Flox for 5'-CTG CAC TCC GAA TTG TGC CTG-3' and YZ198 5'-GAT CCT CGT CTT CAT TGG TAC TG-3', as previously described (Lazorchak et al., 2006b). Briefly, a standard curve is generated using known mixes of flox and deleted alleles at the following ratios: 90%deleted:10%floxed, 75%deleted:25%floxed, 50%deleted:50%floxed, 25%deleted:75%floxed, and 10%deleted:90%floxed. These

standards are created by mixing predetermined numbers of E2A<sup>d/d</sup> (deleted) and E2A<sup>f/f</sup> (flox) Abelson preB cells. DNA was isolated as described above. Once a standard curve is generated by quantitative PCR, samples are normalized to CD14 and plotted on this curve. The primers for CD14 are: for 5'-GCT CAA ACT TTC AGA ATC TAC CGA C-3' and rev 5'-AGT CAG TTC GTG GAG GCC GGA AAT C-3'. The second method used for E2A deletion analysis is a PCR for the flox allele only, using primers neo for and YZ198, at 40 cycles to verify the quantitative PCR results. Neo for: 5'-GCC CAT TCG ACC ACC AAG CG-3'. HEB deletion is analyzed by using the following primers: JW1 5'-CTG GGA CAG AAG TTC AGC ACT TAG TAC -3' and JW2 5'-CAT TCC TAT ACA TCA GCT TCT TGG ACG-3' as described previously (Wojciechowski et al., 2007).

## **2.12 *In vitro* lymphocyte stimulation**

Total LN cells were cultured in RPMI (5% FBS) and 2ng/mL IL-2, with or without 10ng/mL phorbol 12-myristate 13-acetate (PMA) and 1ug/mL ionomycin for 6hrs. 3uM monensin was added for the last 4hrs to prevent release of cytokine.

## **2.13 *Lymph node cell transfer***

Total lymph node cells were harvested from wild-type (CD45.1) and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* (CD45.2) mice and mixed at a 1 to 3 ratio, respectively. Cells were then labeled with 5uM CFSE (Molecular Probes). Approximately 10<sup>7</sup> mixed, CFSE-labeled cells were intravenously injected into Rag2<sup>-/-</sup> deficient hosts.

## **2.14 TCR $\beta$ recombination assay**

The protocol has been described previously (Wojciechowski et al., 2007). Briefly, DNA was isolated from sorted cells by lysis in a Triton lysis buffer as described for deletion analysis. Amplification of rearrangements was done with Platinum Taq polymerase (Invitrogen) and a touchdown PCR program. The following primers were used: V $\beta$ 8 5' consensus 5'-GCA TGG GCT GAG GCT GAT CCA TTA-3' and J $\beta$ 2.7 3' 5'-TGA GAG CTG TCT CCT ACT ATG GAT T-3'. CD14 primers were used as a loading control, and sequences were also given above.

## **2.15 TCR $\alpha$ sequencing analysis**

PCR amplification of cDNA from sorted CD8TCR<sup>+</sup> and CD8TCR<sup>-</sup> *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* LN cells with primers specific for V $\alpha$ 8 and C $\alpha$  yielded a product from any expressed TCR $\alpha$  transcripts using a V $\alpha$ 8 rearrangement. The PCR product was cloned into a PCR4 TOPO vector (Invitrogen) and sequenced. A total of 8 clones were analyzed for each population. The J $\alpha$  gene segment usage and V to J rearrangement frame status were determined from the sequences. The following primer sequences were used: V $\alpha$ 8 5'-CAG ACA GAA GGC CTG GTC AC-3' and C $\alpha$  5'-TGG CGT TGG TCT CTT TGA AG-3'.

## **2.16 Ex vivo DP culture analysis**

For survival assay, sorted CD4<sup>+</sup>CD8<sup>+</sup> DP cells were plated in RPMI media 10% FBS, 55uM 2-mercaptoethanol, L-glut/Pen/Strep, 25mM HEPES. For thymic stromal

culture, thymus was harvested from wild-type CD45.1 congenic mice, cut into approximately 8 pieces and digested for 30min at 37°C with 1mg/mL collagenase 1A (Sigma). A single cell suspension was made and plated for 6hrs. The resulting adherent cell layer was washed to remove most suspension cells. CD45.2<sup>+</sup> CD4<sup>+</sup>CD8<sup>+</sup> DP cells were sorted and plated on the thymic stromal layer the following day in IMDM media 5% FBS, 55uM 2-mercaptoethanol, NaPyr/L-glut/Pen/Strep. FACS plots are pre-gated on 7AAD<sup>-</sup>CD45.1<sup>-</sup>Gr-1<sup>-</sup>Mac-1<sup>-</sup>B220<sup>-</sup> lymphocytes.

### **2.17 Analysis of TCR J $\alpha$ usage**

DNA from CD8 T cells sorted from spleen was used to amplify rearrangement products from V $\alpha$ 8 to J $\alpha$ 22, 49, and 58 as described previously (Abarategui and Krangel, 2006). Briefly, semi-quantitative PCR using 3-fold serial dilution was performed. PCR products were run on an agarose gel and then analyzed by Southern Blot with <sup>32</sup>P-labeled oligonucleotide probes. The following primers were used for PCR amplification: V $\alpha$ 8 5'-CAG ACA GAA GGC CTG GTC AC-3', J $\alpha$ 22 5'-TGT CAG TTG GGT TCC AGA TCC-3', J $\alpha$ 49 5'-GGA ATG ACA GTC AAA CTT GTT CC-3', J $\alpha$ 58 5'-GAC TCA CTG TGA GCT TTG CC-3', and CD14 primers as described above. The following probes were used for Southern Blot analysis: J $\alpha$ 22 5'-TCC AAA GAT GAG TTG CCA GC-3'. J $\alpha$ 49 5'-AGA AGT TCT GGT AAC CCC GTG-3', and J $\alpha$ 58 5'-AGC TTA GAC CCA GTG CCT TG-3'.

## **2.18 Microarray analysis**

### **2.18.1 Pre-proB cell array analysis**

Pre-proB cells were sorted from E2A<sup>ER/ER</sup> and E2A<sup>+/+</sup> (C57BL6) bone marrow after red blood cell lysis and depletion for Mac-1, Gr-1, CD3, and Ter-119 using Dynal Dynabeads (Invitrogen). For sorting, 7AAD<sup>+</sup> dead cells were excluded from gating. Cells were gated based on the following surface marker expression: B220<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>-</sup>Ly6c<sup>-</sup>IgM<sup>-</sup>Mac-1<sup>-</sup>Gr-1<sup>-</sup>CD3<sup>-</sup>Ter119<sup>-</sup>. However, since E2A<sup>ER</sup> mice are on a mixed background, not all mice carry the NK1.1 marker, or may be heterozygous for this C57BL6 strain-specific marker. For E2A<sup>ER/ER</sup> cells, approximately  $1-1.5 \times 10^4$  pre-proB cells were plated per well in round-bottom 96-well plates in hormone free 5% FBS RPMI supplemented with 10ng/mL IL-7, with or without 1uM Tamoxifen for 12hr culture. For E2A<sup>+/+</sup> cells, approximately  $1-1.5 \times 10^4$  pre-proB cells were either harvested immediately after the sort or cultured in hormone free 5%FBS RPMI supplemented with 10ng/mL IL-7 for 12hr culture. Sorted and cultured cells were harvested and collected for SuperAmp lysis per manufacturer's instructions (Miltenyi Biotec). This sort and culture was performed in duplicate experiments. Agilent Microarray service was performed by Miltenyi Biotec using the whole mouse genome, 4 x 44K, Two-color microarray with SuperAmp Service. The E2A<sup>ER/ER</sup> DMSO only control treated cultures were Cy3-CTP labeled and E2A<sup>ER/ER</sup> Tamoxifen treated cultures were Cy5-CTP labeled. The E2A<sup>+/+</sup> sorted cells were Cy3-CTP labeled and the E2A<sup>+/+</sup> cultured cells were Cy5-CTP labeled.

Two comparisons were performed, each in duplicate: E2A<sup>ER/ER</sup> tamoxifen vs E2A<sup>ER/ER</sup> DMSO cultures and E2A<sup>+/+</sup> cultured cells vs E2A<sup>+/+</sup> sorted cells. Data filtering was performed using GeneSpring software. Genes that do not have unigene identification were excluded from Tables 3 and 4.

### 2.18.2 DP thymocyte array analysis

CD4<sup>+</sup>CD8<sup>+</sup> DP cells were sorted from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* thymus. The same CD4<sup>hi</sup>CD8<sup>hi</sup> gate was used for both *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* sorting, to assure analysis of populations expressing similar CD4 and CD8 levels. Independent sorts from 2 mice per genotype were done. RNA was extracted using RNeasy QIAGEN kit with DNase I step following manufacturer's protocol. Array analysis was performed by the Duke Microarray Core Facility (<http://microarray.genome.duke.edu/services/spotted-arrays/protocols>). Briefly, one round of amplification was performed, RNA samples were labeled with Cy3 or Cy5 dyes, and samples were hybridized to the Mouse Operon oligo set 4.0 Chip. Two comparisons were performed: *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* to a Universal Mouse Reference RNA (Stratagene) and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* to this same reference, each done in duplicate. Data filtering and statistical analysis were performed using GeneSpring software. *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* versus *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* comparisons were done using averages of the duplicates, filtering out any genes with > 2

s.d. within the groups. Genes that have neither ensembl nor unigene identification are excluded from Tables 6 and 7.

## **2.19 Statistical analysis**

Statistical significance was assessed by the two-tailed Student's *t*-test.

### **3. Dissecting E2A function in B lineage commitment with an E2A inducible mouse model**

#### **3.1 Introduction**

E2A, the best characterized E-protein family member in mammals, has been shown to have stage specific roles in cell differentiation, lineage commitment, proliferation, and survival. However, due to the complexity of E2A function, it is often difficult to separate these roles using conventional genetic approaches. Here, we have developed a new genetic model in which E2A protein activity can be reversibly regulated at endogenous levels. In addition, we have used this system to begin taking a closer look at E2A function as cells commit to the B cell lineage.

We have established an inducible E2A mouse model by inserting a tamoxifen-responsive region of the estrogen receptor (ER) ligand binding domain at the carboxyl end of *tcfe2a*, resulting in the production of E2AER fusion proteins. The use of tamoxifen inducible ER fusion proteins in mouse genetics has already been established as a valuable tool, especially with the vast use of the Cre recombinase-ER fusion protein for inducible gene knockout in mice (Feil et al., 1996; Hayashi and McMahon, 2002; Schwenk et al., 1998; Vooijs et al., 2001). In addition, ER fusion with a variety of transcription factors has also been successfully employed for analysis of gene expression. For example, MyoD-ER fusion proteins have been expressed by viral transduction in mouse embryonic fibroblasts for *in vitro* study of MyoD gene regulation (Bergstrom et al., 2002) and in mouse fibroblasts for analysis of MyoD activation *in vivo*

post transplantation of transduced cells into recipient animals (Kimura et al., 2008). However, we don't know how useful the ER system will be in live animals when targeting an endogenous locus. Our E2A<sup>ER</sup> system now introduces an ER fusion with a bHLH transcription factor into the mouse genome for analysis of an endogenously expressed protein. Since E2A is the common factor for multiple lineage specific HLH transcription factor dimers, the E2A<sup>ER</sup> model may benefit a range of developmental biology research areas.

E2A<sup>ER</sup> mice and E2AER protein function are analyzed here in the context of B cell development. A block in B cell development at the pre-proB cell stage, prior to B lineage commitment, has been characterized by previous E2A-deficient mouse models (Bain et al., 1994; Zhuang et al., 1994). A rescue in B cell development from pre-proB cells upon tamoxifen treatment would be a stringent test to verify inducible E2AER function. Following validation of the system, initial E2A-mediated events required for pre-proB to proB development can then be investigated. Previous analysis of E2A function during B lineage commitment has been limited mainly to studies in cell lines and rescue of E2A-deficient progenitors by over-expression experiments (Greenbaum et al., 2004; Ikawa et al., 2004; Kee and Murre, 1998; Seet et al., 2004). With the E2A<sup>ER</sup> system, endogenously expressed E2AER protein can be activated in primary cells to restore E2A function. Analysis of the initial events downstream of E2A activation at the pre-proB cell stage is

expected to provide us with novel targets and kinetic analysis of B lineage gene activation.

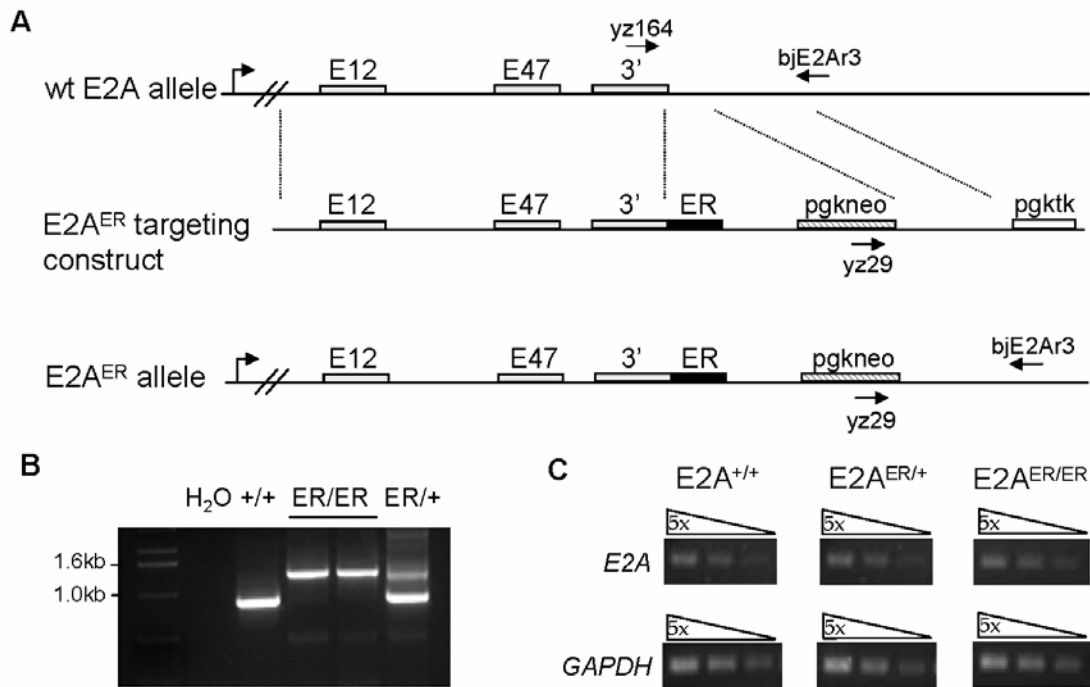
In this study, we provide the initial characterization of the E2A<sup>ER</sup> system. E2A<sup>ER</sup> activity is rapidly induced upon tamoxifen treatment and can be reversibly regulated by tamoxifen withdrawal. Activated E2A<sup>ER</sup> protein is able to rescue and support B cell development from E2A<sup>ER</sup> pre-proB cells in an *ex vivo* culture system. In addition, we have performed microarray analysis using tamoxifen-treated E2A<sup>ER</sup> pre-proB cells to search for the initial E2A target genes required for B lineage commitment and further development. This analysis has identified several genes that have yet to be described as E2A targets or critical factors in pre-proB cells.

## **3.2 Results**

### **3.2.1 Generation of E2A<sup>ER</sup> mice**

E2A<sup>ER</sup> mice were generated by using a knock-in strategy for tamoxifen-inducible E2A function. The tamoxifen-responsive ligand binding domain of the mouse ER (Zhao et al., 2001) was inserted at the carboxyl end of *tcfe2a* to produce E2A<sup>ER</sup> mice (Figure 5A,B). With this targeting strategy, similar to that used for the E2A<sup>GFP</sup> strain previously developed in our lab (Zhuang et al., 2004), both alternatively spliced products of the *tcfe2a* gene, E12 and E47, are translated as ER fusion proteins. Initial characterization of the E2A<sup>ER</sup> allele indicated normal expression levels of E2A in the presence of the ER insertion (Figure 5C). Previous study of E2A knockout mice demonstrated stunted

growth and high lethality rate of homozygous animals within the first few weeks after birth (Bain et al., 1994; Zhuang et al., 1994). In contrast, the E47<sup>bm</sup> strain, expressing a dominant negative form of E47, was originally described as indistinguishable from wild type litter mates in size and survival (Zhuang et al., 1998). However, this work was analyzing mice on a mixed genetic background and upon backcrossing to C57BL/6, the E47<sup>bm</sup> homozygous mice became smaller in size and demonstrated the high lethality rate like that shown with the knockout animals (unpublished data). E2A<sup>ER</sup> homozygous animals also exhibit stunted growth and reduced survival. Fortunately, the lethality rate in our experience is less severe in E2A<sup>ER</sup> litters than that observed with our E2A knockout and dominant negative strains (Table 2). However, we do not know if this slight increase in postnatal survival is due to the presence of the E2A<sup>ER</sup> protein or because our E2A<sup>ER</sup> mice are currently on a mixed background.



**Figure 5: Generation of E2A<sup>ER</sup> mice**

(A) Targeting strategy for the generation of E2A<sup>ER</sup> mice. The 3' region of the mouse *tcf2a* gene was used for targeting. Gene direction, the E47, E12, and 3' exons (gray boxes), and inserted tamoxifen-responsive estrogen receptor ligand binding domain (ER, black box) are shown. Regions mediating homologous recombination are denoted by dotted lines. The selection markers pgkneo and pgktk are located as shown. (B) Genotyping PCR of E2A<sup>+/+</sup>, E2A<sup>ER/+</sup>, and E2A<sup>ER/ER</sup> DNA using primers yz164, yz29, and bjE2Ar3 as shown in (A), that yield a 0.9kb wild-type allele and 1.2kb mutant allele. (C) RT-PCR of cDNA acquired from E2A<sup>+/+</sup>, E2A<sup>ER/+</sup>, and E2A<sup>ER/ER</sup> thymus RNA. E2A and GAPDH (control) expression was detected by semi-quantitative PCR of 5-fold serial dilutions. E2A primers E2A f1 and E2A r1 were used to amplify a region spanning common exons at the 5' end of E2A cDNA.

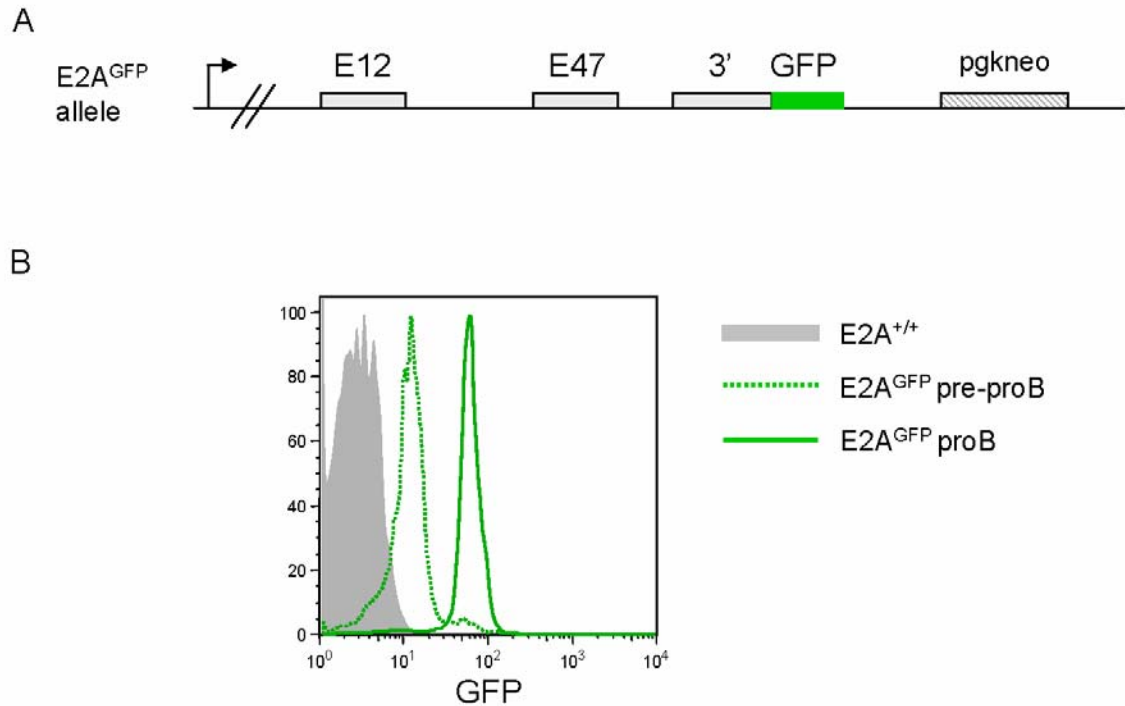
**Table 2: Characterization of E2A gene-targeted mice**

	<b>E2A<sup>+/+</sup> wild type</b>	<b>E2A<sup>gal</sup> knockout null allele</b>	<b>E2A<sup>E47bm</sup> knockin basic region mutation</b>	<b>E2A<sup>GFP</sup> knockin C-term fusion</b>	<b>E2A<sup>ER</sup> knockin C-term fusion</b>
<b>E2A activity</b>	normal	null	dominant negative	normal	tamoxifen inducible
<b>Growth</b>	normal	stunted	stunted	normal	stunted in most
<b>Viability</b>	normal	high rate lethality	high rate lethality	normal	moderate rate lethality
<b>B cell development</b>	normal	block at pre-proB to proB stage	block at pre-proB to proB stage	normal	block at pre-proB to proB stage

### **3.2.2 B cell developmental block in E2A<sup>ER</sup> mice**

The pre-proB cell stage is an intermediate stage as lymphoid progenitors develop into committed proB cells. Pre-proB cells can be characterized by the expression of B220 and CD43 and the absence of CD19 expression. As pre-proB cells transition to the proB cell stage, CD19 expression is induced and cells undergo commitment to the B cell lineage. E2A is critical for this transition, as demonstrated by the block in development at the pre-proB cell stage in E2A-deficient animals (Bain et al., 1994; Bain et al., 1997b; Zhuang et al., 1994). Analysis of E2A<sup>GFP</sup> mice displays the up-regulation of E2A protein levels from the pre-proB to proB stage (Figure 6) (Zhuang et al., 2004). This increase in E2A expression is likely critical for E2A's regulation of the B cell lineage gene expression profile given the importance of E2A gene dosage. For example, elimination of one copy

of E2A has been shown to greatly reduce the number of proB cells (Zhuang et al., 1996; Zhuang et al., 1994).



**Figure 6: Up-regulation of E2A protein levels during pre-proB to proB cell stage development**

**(A)** E2A<sup>GFP</sup> allele (Zhuang et al., 2004). An E2A-GFP fusion protein is produced from this allele. Therefore, GFP expression can be used to monitor E2A protein levels. **(B)** E2A<sup>GFP/GFP</sup> and E2A<sup>+/+</sup> control bone marrow was stained for B220, CD43, and CD19 surface expression. Cells are pre-gated on lymphocytes. GFP expression is shown for E2A<sup>+/+</sup> B220<sup>+</sup>CD43<sup>+</sup> (pre-proB + proB) control cells, and E2A<sup>GFP/GFP</sup> pre-proB (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>) and proB (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>+</sup>) compartments.

Investigation of B cell development in E2A<sup>ER</sup> mice reveals a block at the pre-proB cell stage, similar to that seen in the E2A-mutant strain E47<sup>bm</sup> (Figure 7) (Zhuang et al.,

1998). Occasionally we do observe a population of CD19<sup>+</sup> B cells in the bone marrow of E2A<sup>ER</sup> mice (Figure 8), but these incidences of leaky B cell development are rare and most cells fail to progress past the preB cell stage, when E2A is known to be critical for Igκ recombination (Inlay et al., 2004). Even though E2A has been suggested to influence proliferation in developing B cells (Borghesi et al., 2005; Herblot et al., 2002; Seet et al., 2004; Zhao et al., 2001), no significant difference in expansion at the pre-proB cell stage was observed by BrdU analysis of E2A<sup>ER</sup> and wild-type mice (Figure 9).

**Figure 7: B cell developmental block at the pre-proB cell stage in E2A<sup>ER</sup> mice**

**Representative staining of bone marrow cells from E2A<sup>+/+</sup>, E2A<sup>ER/+</sup>, E2A<sup>ER/ER</sup>, E2A<sup>E47bm/+</sup>, and E2A<sup>E47bm/E47bm</sup> mice. Cells are pre-gated on 7AAD<sup>-</sup> lymphocytes and relative percentages are given in each plot. Cells from the CD43<sup>+</sup>B220<sup>+</sup> gate are displayed in histograms analyzing CD19 expression. Pre-proB (CD19<sup>-</sup>) and proB (CD19<sup>+</sup>) cell percentages are shown.**

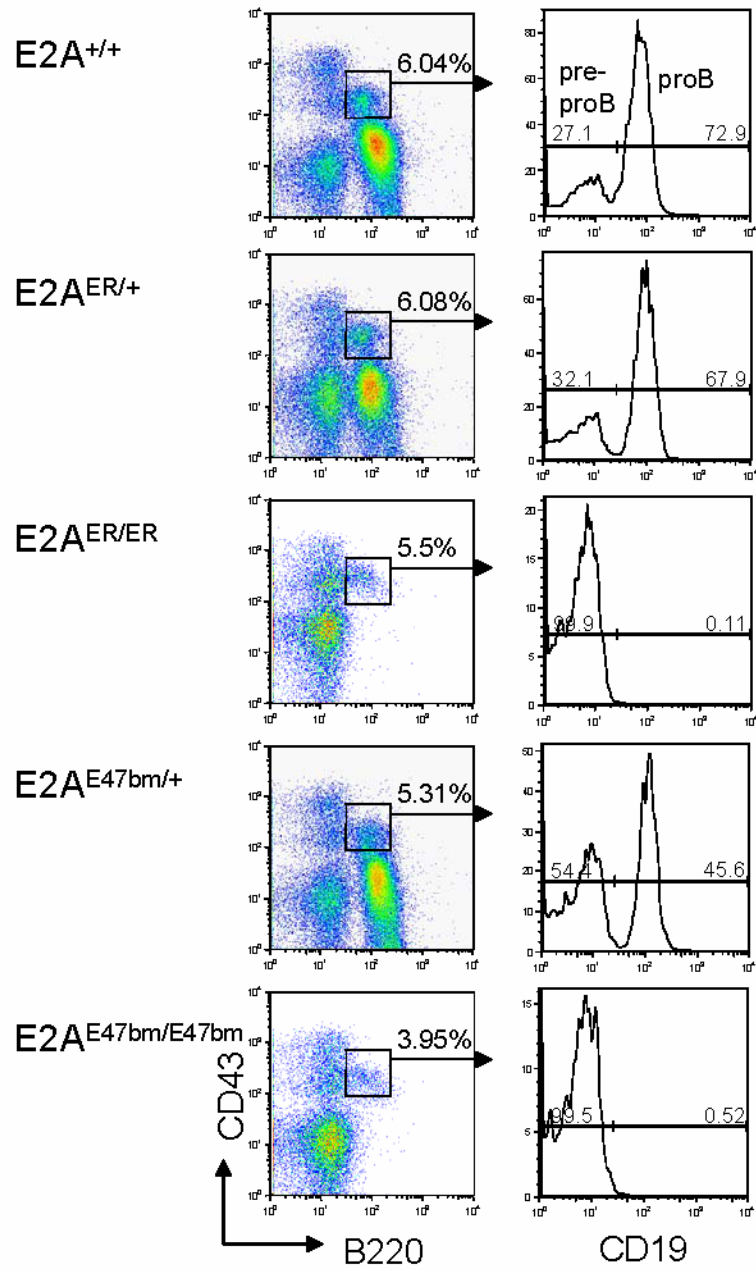


Figure 7: B cell developmental block at the pre-proB cell stage in E2A<sup>ER</sup> mice

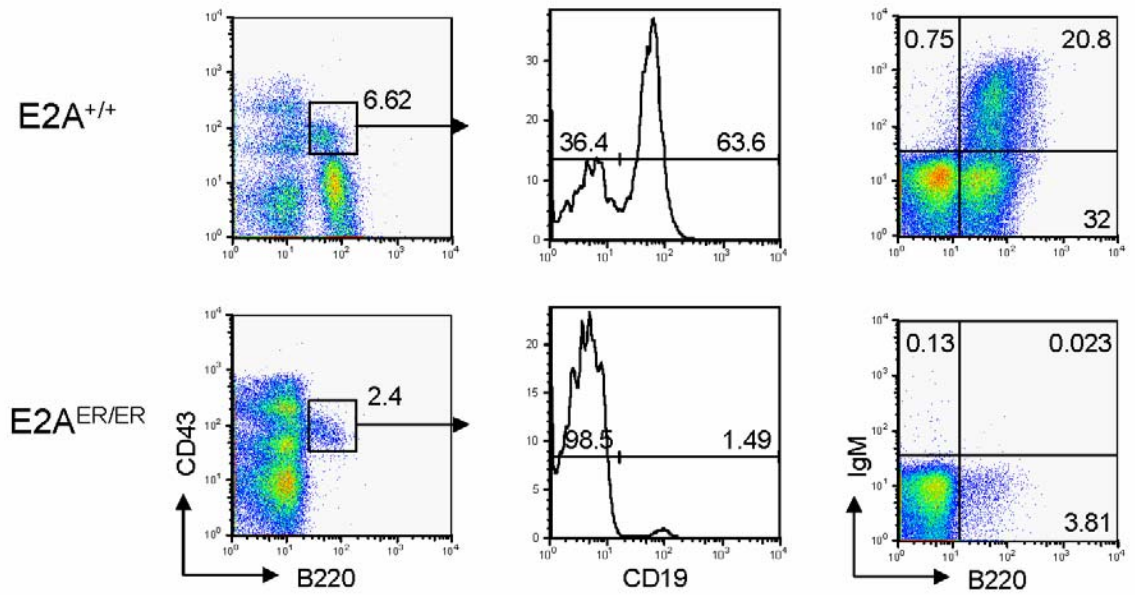
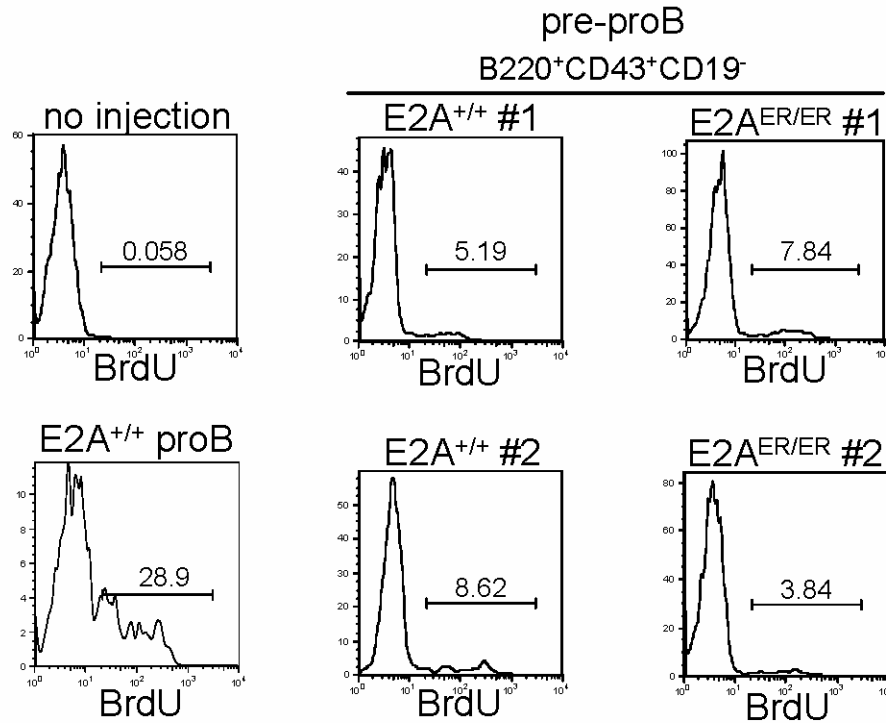


Figure 8: Rare CD19<sup>+</sup> B cell population detected in a few  $E2A^{ER}$  mice

Staining of bone marrow from  $E2A^{ER}$  and  $E2A^{+/+}$  control mice for B220, CD43, CD19, and IgM expression. All plots are pre-gated on 7AAD<sup>-</sup> lymphocytes. Histograms are pre-gated on B220<sup>+</sup>CD43<sup>+</sup> cells as shown. Relative percentages are displayed.



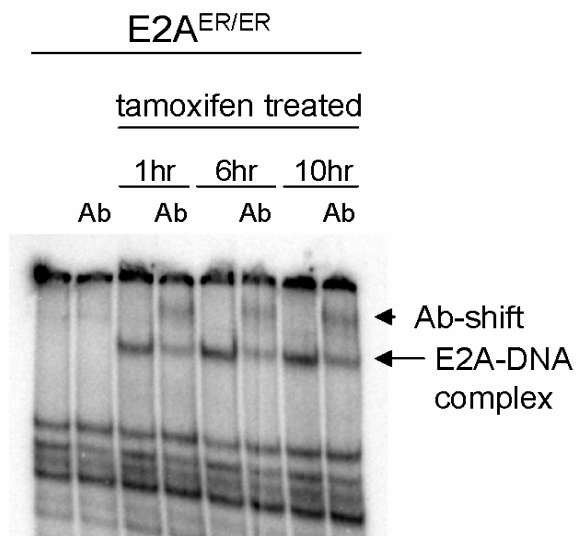
**Figure 9: *In vivo* BrdU labeling suggests similar expansion of E2A<sup>ER</sup> pre-proB cells compared to E2A<sup>+/+</sup> pre-proB cells**

Two mice from each genotype, E2A<sup>ER</sup> and E2A<sup>+/+</sup> control, were IP injected with 1mg BrdU and analyzed 4hrs post injection. Bone marrow was stained for B220, CD43, and CD19 surface expression, then processed to analyze BrdU labeling. All plots are pre-gated on lymphocytes. Graphs display percent BrdU<sup>+</sup> cells within the pre-proB population (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>) as labeled. An E2A<sup>ER</sup> mouse receiving no BrdU injection was used as a negative control (plot displays pre-proB cells). The proB population (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>+</sup>) from BrdU injected E2A<sup>+/+</sup> #2 is shown as a positive control for BrdU incorporation.

### 3.2.3 Rapid and reversible regulation of E2AER activity

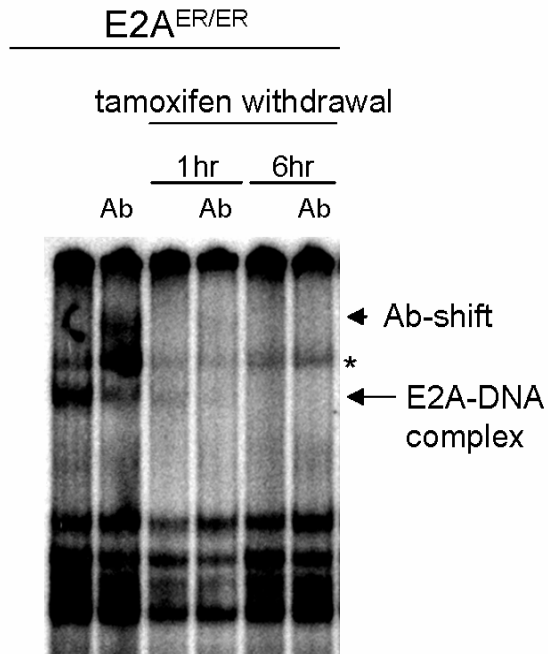
For analysis of E2AER DNA binding activity, we chose to derive Abelson transformed preB cells from E2A<sup>ER</sup> bone marrow (Rosenberg and Kincade, 1994). Nuclear extracts from E2A<sup>ER</sup> Abelson cells were used to conduct electrophoretic gel shift analysis of E2AER binding to an E2A binding sequence,  $\mu$ E5. In the absence of tamoxifen treatment, no DNA binding activity was observed from the E2AER protein (Figure 10). Together, the block in B cell development and lack of E2AER DNA binding activity suggest that the E2A<sup>ER</sup> mouse model functions as an E2A-deficient system in the absence of ligand.

Tamoxifen treatment of E2A<sup>ER</sup> Abelson cells resulted in rapid E2AER DNA binding activity within 1hr of treatment (Figure 10). The specificity of the protein binding to the  $\mu$ E5 probe was verified by using an anti-E2A antibody that effectively super-shifted the protein/DNA complex. The effect of tamoxifen withdrawal was then tested by washing tamoxifen-treated cells and growing them in the absence of tamoxifen for 1 and 6hr time points. Loss of E2AER DNA binding activity was seen within 6hrs of tamoxifen withdrawal (Figure 11), indicating relatively fast reversibility of E2A function. These results demonstrate that the E2A<sup>ER</sup> model can be used not only as an inducible model, but this system may also be valuable for providing a tightly regulated window of E2A activity.



**Figure 10: Induction of E2A<sup>ER</sup> DNA binding activity**

Nuclear extracts from Abelson-transformed E2A<sup>ER</sup> preB cells cultured with and without tamoxifen for 1, 6 and 10hr were analyzed by gel shift for E2A DNA-binding using a  $\mu$ E5 probe. Anti-E2A antibody was used to demonstrate specificity (Ab). E2A-DNA complexes (arrow) and antibody-shifted complexes (arrow head) are indicated.



**Figure 11: Reversible regulation of E2A<sup>ER</sup> DNA binding activity by tamoxifen withdrawal**

Nuclear extract from Abelson-transformed E2A<sup>ER</sup> preB cells cultured with tamoxifen and upon withdrawal of tamoxifen for 1hr and 6hr was analyzed by gel shift for E2A DNA-binding using the  $\mu$ E5 probe. Anti-E2A antibody was used to demonstrate specificity (Ab). E2A-DNA complexes (arrow) and antibody-shifted complexes (arrow head) are indicated. \*Non-specific band

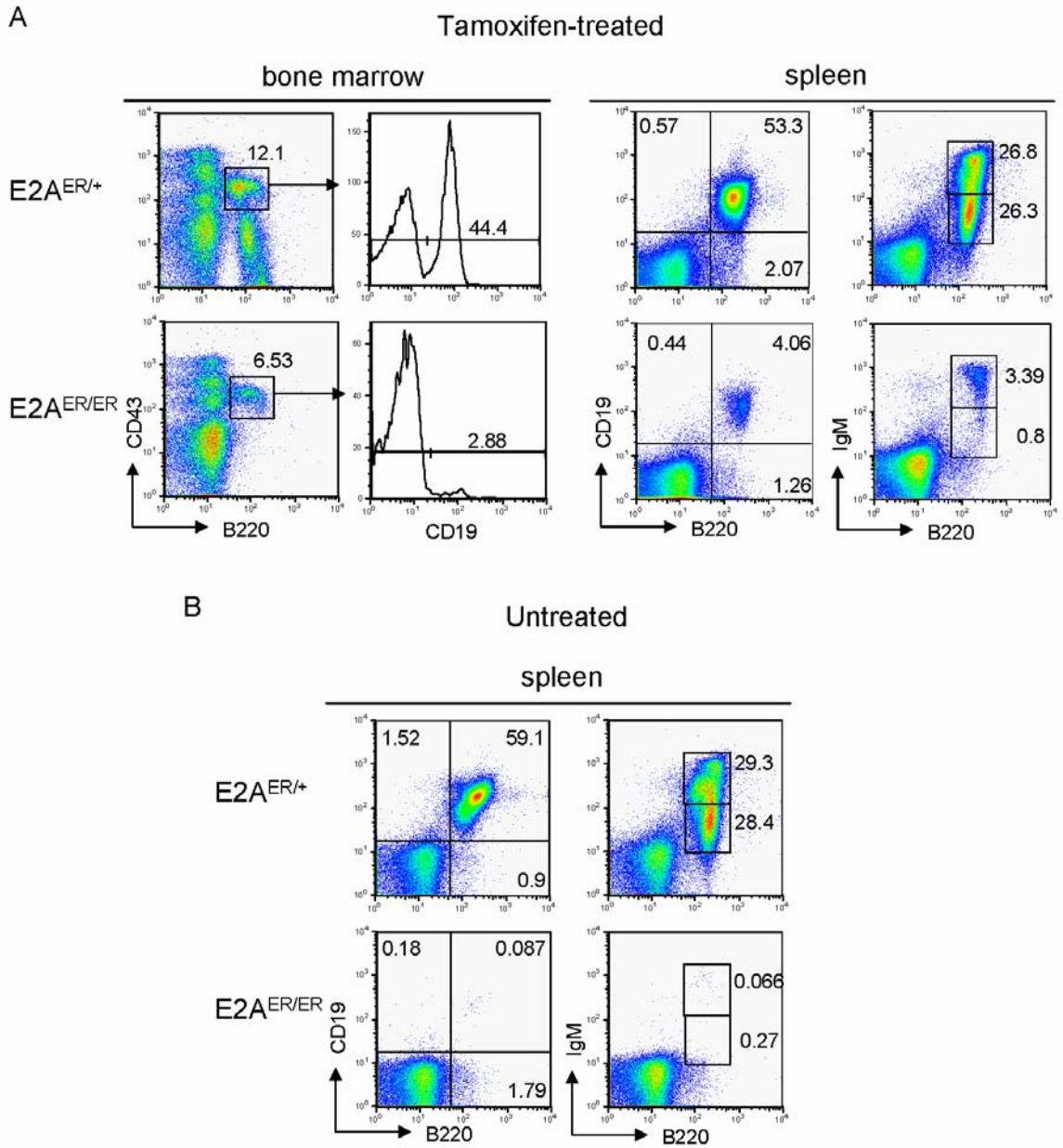
### 3.2.4 Induction of E2AER activity supports B cell development

#### 3.2.4.1 Inefficient B cell development upon *in vivo* tamoxifen treatment

We first tested for a functional outcome of E2A induction by *in vivo* tamoxifen treatment of E2A<sup>ER</sup> mice followed by analysis of B cell populations in the bone marrow and spleen. All *in vivo* treatment efforts, including IP injection (data not shown) and treatment in drinking water, unfortunately resulted in low efficiency rescue of B cell development. The emergence of B cells in tamoxifen-treated animals was rarely great enough to determine if the resulting B cells were generated in response to the tamoxifen treatment or were simply the incidence of leaky B cell development described above. The most significant recovery of B cells observed from *in vivo* treatment, which resulted from a 27 day tamoxifen treatment, was still considerably less than the B cell population in control mice (Figure 12). However, the presence of IgM<sup>+</sup> B cells in the spleen does suggest that tamoxifen-dependent B cell development occurred *in vivo*.

#### Figure 12: Inefficient rescue of B cell development upon *in vivo* tamoxifen treatment of E2A<sup>ER</sup> mice

(A) Two month old E2A<sup>ER/ER</sup> and E2A<sup>ER/+</sup> control mice were treated with tamoxifen in their drinking water for 27 days. Indicated tissues were stained as shown. All plots are pre-gated on 7AAD<sup>-</sup> lymphocytes. Histograms are pre-gated on B220<sup>+</sup>CD43<sup>+</sup> cells as shown. Relative percentages are displayed. (B) Representative spleen staining from age-matched non-treated E2A<sup>ER/ER</sup> and E2A<sup>ER/+</sup> mice.



**Figure 12: Inefficient rescue of B cell development upon *in vivo* tamoxifen treatment of E2A<sup>ER</sup> mice**

#### 3.2.4.2 *Ex vivo* tamoxifen treatment effectively rescues B cell development

In contrast to *in vivo* tamoxifen treatment, treatment in an *ex vivo* B cell culture system effectively rescued B cell development. Sorted E2A<sup>ER</sup> pre-proB cells were cultured in hormone-free media on an S17 stromal layer in the presence of IL-7 with or without tamoxifen. Control DMSO treated E2A<sup>ER</sup> pre-proB cells failed to develop efficiently into CD19<sup>+</sup> B cells over the course of 5 days, whereas tamoxifen treated E2A<sup>ER</sup> pre-proB cells effectively gave rise to CD19<sup>+</sup> B cells (Figure 13A). Interestingly, the kinetics of B cell development from tamoxifen treated E2A<sup>ER</sup> pre-proB cells appeared delayed compared to that of control cells. In addition to using CD19 expression to validate the rescue of B cell development, we analyzed Pax5 expression throughout the 5 day culture. Consistent with CD19 expression, induction of Pax5 expression was observed in tamoxifen treated E2A<sup>ER</sup> pre-proB cells, also appearing delayed compared to control cultures (Figure 13B). In addition to providing functional proof of induced E2A activity, these results demonstrate that restored E2A function can rescue B cell development from the pre-proB cell stage.

**Figure 13: Tamoxifen treatment restores B cell development from E2A<sup>ER</sup> pre-proB cells**

Sorted E2A<sup>ER/+</sup> and E2A<sup>ER/ER</sup> pre-proB cells were plated in hormone-free media on S17 stromal cells on Day 0 in the presence of IL-7 with tamoxifen or DMSO (untreated control). (A) Cells are pre-gated on 7AAD<sup>-</sup>B220<sup>+</sup> lymphocytes. Percents of CD19<sup>+</sup> cells on Day 1, 3, and 5 are displayed. Data are representative of 3 independent experiments. (B) Expression of Pax5 was analyzed by quantitative RT-PCR from RNA collected from Day 1, 3, and 5 cultures shown in (A). Samples were normalized to the expression of GAPDH. Graphed results are means from triplicate runs (n=3) with error bars representing standard error of the mean (SEM).

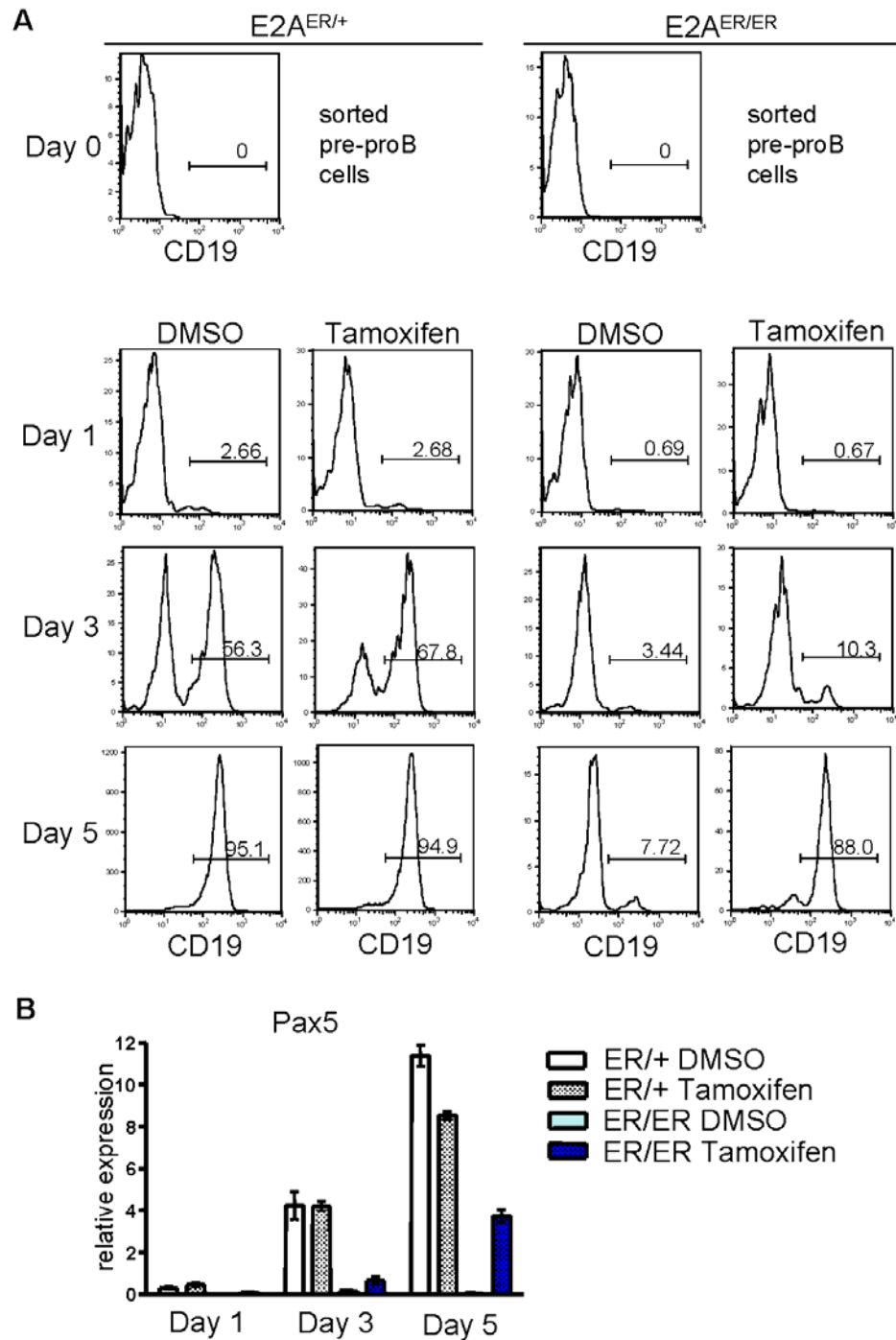


Figure 13: Tamoxifen treatment restores B cell development from  $E2A^{ER}$  pre-proB cells

### 3.2.5 Identification of novel E2A targets during B lineage commitment

Once we established that B cell development could be rescued from the pre-proB cell stage using the E2A<sup>ER</sup> system, we next wanted to identify the initial genes altered upon activation of E2AER activity to allow these pre-proB cells to begin commitment to the B cell lineage. E2A<sup>ER</sup> pre-proB cells provide a population of cells that are poised for B cell development, and E2AER function can be rapidly and simultaneously turned on within this population to instruct development to the B cell lineage. In order to get a broad view of E2A gene regulation upon tamoxifen treatment of E2A<sup>ER</sup> pre-proB cells, we performed microarray analysis using a whole mouse genome array. Pre-proB cells were sorted from either wild-type or E2A<sup>ER</sup> mice and cultured in hormone free media supplemented with IL-7. Two comparisons were performed. For wild-type cells, cells after culture were compared to cells immediately isolated after sorting. This comparison should identify any gene expression changes due to the *ex vivo* culture. For E2A<sup>ER</sup> cells, control-treated cultured cells were compared to tamoxifen-treated cultured cells. This comparison should identify gene expression changes due to tamoxifen treatment of E2A<sup>ER</sup> pre-proB cells. Each experiment was done in duplicate (A and B).

For the initial analysis, a 12hr culture/treatment time point was chosen. Even though this time span is too long to limit our analysis to direct E2A targets, we wanted to ensure a broad range of genes were significantly altered. In fact, a great number more genes were either up-regulated or down-regulated than anticipated within 12hrs of

tamoxifen treatment (Table 3). However, a similar number were identified from wild-type cells simply cultured in the presence of IL-7 for 12hrs.

**Table 3: Number of genes demonstrating greater than 2 fold change, p-value  $\leq 0.05$**

<b>Data set (s)</b>	<b># genes up-regulated</b>	<b># genes down-regulated</b>
WT A	3,616	3,870
WT B	4,494	4,737
ER A	4,906	5,534
ER B	4,291	4,351
WT A & B	876	1,008
ER A & B	958	1,130
WT A,B & ER A,B	43	52

Given the large number of genes demonstrating a change in expression after culture and upon tamoxifen treatment, we chose to narrow our analysis by concentrating first on the genes altered in E2A<sup>ER</sup> pre-proB cells upon tamoxifen treatment. To further simplify the analysis we focused on the list of genes up-regulated in both A and B duplicate experiments (958 genes) and the list of genes down-regulated in both A and B duplicate experiments (1,130 genes). A candidate gene list was formed by identifying genes that fell into one of the following groups: genes that were known to function in B cells, genes that were expressed in bone marrow or B cell populations, transcription factors known or suggested to regulate developmental programs or key factors, and previously suggested E2A target genes. The list formed was by no means

inclusive, but provided a more manageable list of genes to investigate as potential E2A targets during B lineage commitment. Genes were then eliminated if expression was not detectable by RT-PCR in wild-type bone marrow. This filtering process left us with our initial short list of candidate genes to further investigate (Table 4). Within this list, there are two positive controls, genes encoding TdT and VpreB, which are known E2A target genes during B cell development (Greenbaum and Zhuang, 2002). The up-regulation of both of these genes upon tamoxifen treatment gave us confidence in the microarray results from these experiments. In addition to these two genes, this list contains two other genes up-regulated and three genes down-regulated upon tamoxifen treatment. Since many of the gene expression changes identified by this microarray analysis could have resulted from E2A-independent mechanisms, for example IL-7-mediated effects, we hope to first identify which of these genes are true E2A targets and which are important during B cell development.

**Table 4: Microarray identified potential E2A target genes during pre-proB to proB development**

Gene	Fold Change Tamox/DMSO A	Fold Change Tamox/DMSO B	Description
Dntt	39.11	5.42	TdT, V(D)J recombination role
VpreB	2.823	3.68	surrogate light chain component
Numb	19.68	10.3	Notch antagonist
Trib2	12.3	2.41	tribbles homolog 2 (Drosophila), inhibitor of CEBP $\alpha$
Pias3	0.03	0.39	Stat3 inhibitor
Stat5b	0.111	0.309	essential for B cell development
Stat6	0.324	0.41	gene regulation during B cell development

Because E2A is up-regulated from the pre-proB to the proB stage (Figure 6), we chose to analyze gene expression during this transition in wild-type cells to see if there were any correlations with the microarray results. In addition, to look for an E2A-dependent change in expression, we analyzed the E2A<sup>ER</sup> Abelson cells upon tamoxifen treatment. So far, Trib2 has emerged as an interesting target to further pursue. Trib2 expression, up-regulated in the microarray, was also up-regulated from the pre-proB to the proB cell stage in wild-type animals (Figure 14A). Additionally, Trib2 expression was enhanced upon tamoxifen treatment of E2A<sup>ER</sup> Abelson cells (Figure 14B). Trib2 is one of three members of the mammalian tribbles protein family that has been studied for its role in hematopoiesis by over-expression in hematopoietic progenitors (Keeshan et al., 2006). In this study, Trib2 was identified as an oncoprotein that is capable of

inhibiting the function of CEBP $\alpha$ . Lethally irradiated mice reconstituted with Trib2-expressing hematopoietic progenitors exhibit defects in myeloid development and develop acute myelogenous leukemia (AML). However, lymphoid development in these mice was reported to be unperturbed. It is not known if Trib2 plays a role in early B cell development, but this gene was of interest given its negative regulation of CEBP $\alpha$ , a critical factor for myeloid lineage development (Hsu et al., 2006; Yamanaka et al., 1998). Additional experiments to test Trib2 and other candidate include chromatin immunoprecipitation analysis to test for E2A binding and manipulation of gene expression to determine if these genes are critical during development of the B cell lineage.

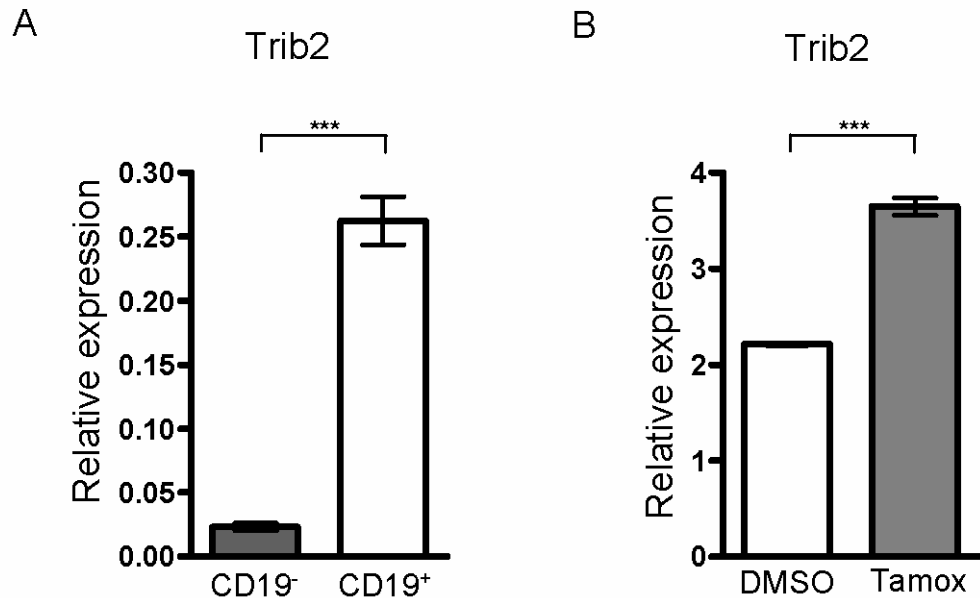


Figure 14: Trib2 as a potential E2A target during early B cell development

(A) Trib2 expression increases from pre-proB to proB cell stage. Expression of Trib2 was analyzed by quantitative RT-PCR from RNA collected from sorted wild-type B220<sup>+</sup>CD43<sup>+</sup> populations: CD19<sup>-</sup> (pre-proB) and CD19<sup>+</sup> (proB). Samples were normalized to the expression of GAPDH. Graphed results are means from triplicate runs from 3 independent samples (n=9). (B) Trib2 expression is enhanced upon E2A induction. Expression of Trib2 was analyzed by quantitative RT-PCR from RNA collected from E2A<sup>ER</sup> Abelson cells treated with DMSO (control) or Tamoxifen for 24hr. Samples were normalized to the expression of GAPDH. Graphed results are means from triplicate runs (n=3). Error bars represent SEM. \*\*\*p $\leq$ 0.001.

### 3.3 Discussion

In addition to identifying potential novel E2A target genes during B cell development, the E2A<sup>ER</sup> system more importantly provides an improved method for

analyzing E2A function. Previous analysis using over-expression of E2AER fusion proteins by retroviral transduction has already demonstrated the value of inducible E2A activity (Greenbaum et al., 2004; Ikawa et al., 2004; Lazorchak et al., 2006a; Sayegh et al., 2003; Zhao et al., 2001). However, toxicity is often a problem with high levels of E2A in the cell (Engel and Murre, 1999; Zhao et al., 2001), and retroviral transduction is not ideal for all cell types. In addition, the changes in gene expression detected upon over-expression of E2A may not always be representative of endogenous E2A function. Therefore, the E2A<sup>ER</sup> mouse model is an attractive system for studying gene regulation and other E2A-regulated events in potentially any E2A-expressing cell type at a more physiological level.

Here we demonstrate the rapid induction of E2AER DNA binding activity upon tamoxifen treatment and its potential for reversible function upon tamoxifen withdrawal. The ability to tightly control E2A activity allows for kinetic analysis of downstream events. Analysis of B cell development in our *ex vivo* culture system demonstrates a rescue from the pre-proB cell stage, but suggests delayed kinetics from E2A<sup>ER</sup> pre-proB cells compared to B cell development from control pre-proB cells. This result suggests that even though E2A<sup>ER</sup> and wild-type pre-proB cells are phenotypically similar, E2A<sup>ER</sup> pre-proB cells may not have equivalent developmental potential or may require additional cellular changes prior to entering the B cell lineage. It is possible that E2A-regulated events normally occurring earlier in development, as suggested in the

LMPP and the CLP stages (Borghesi et al., 2005; Dias et al., 2008), are occurring upon restored E2A function in pre-proB cells and requiring these cells to “catch up” with their wild-type counterparts prior to progressing to the next stage. Alternatively, the developing tamoxifen-treated E2A<sup>ER</sup> B cells may not expand as rapidly as wild-type cells upon commitment to the B cell lineage. Further investigation of tamoxifen treatment of E2A<sup>ER</sup> pre-proB cells may help to identify why the delayed detection of CD19<sup>+</sup> B cells is observed.

The delay of Pax5 expression in the *ex vivo* culture (Figure 13) and the absence of EBF1 up-regulation in the microarray (data not shown) suggest that these are not immediate events upon restored E2A activity. There are likely additional, novel E2A targets activated or repressed prior to up-regulation of EBF1 and Pax5 that function to direct cells to the B cell lineage. We hope to use the list of genes identified from our microarray analysis of tamoxifen treated E2A<sup>ER</sup> pre-proB cells to begin identifying some of these early targets. For example, given its known function to inhibit the myeloid factor CEBP $\alpha$ , Trib2 could be an E2A target gene up-regulated to repress alternative lineages as cells are directed to a B cell fate. However, additional experiments will be necessary to test this hypothesis.

While *in vivo* tamoxifen treatment was not efficient for restoring B cell development, *in vivo* treatment may still be useful for analysis of E2A function in other cell lineages. It is possible that the tamoxifen concentration in the bone marrow is not

high enough for efficient binding to the ER. However, we suggest two alternative reasons for ineffective rescue of *in vivo* B cell development. First, progression through several stages of lymphocyte development is known to be dependent on proper E-protein dosage (Bain et al., 2001; Barndt et al., 2000; Herblot et al., 2002; Quong et al., 2004; Zhuang et al., 1996; Zhuang et al., 1994), and it is possible that the required E2A threshold is not maintained by our *in vivo* treatment methods for rescue of B cell development. Second, E2A has been suggested to be important for B cell survival (Lazorchak et al., 2006b). Therefore, it is conceivable that even if B cells are rescued by tamoxifen treatment, they may be lost if E2A activity is not maintained throughout the treatment. The E2A<sup>ER</sup> system may instead be more valuable for *in vivo* study of T cell development, or other lineages expressing multiple E-protein family members. Since E-proteins demonstrate many redundant functions, T cell development is not completely blocked in E2A-deficient mice due to the presence of HEB (Bain et al., 1999; Barndt et al., 2000; Wojciechowski et al., 2007; Zhuang et al., 1996). Because T cells are fully developed in E2A-deficient mice, *in vivo* tamoxifen treatment of E2A<sup>ER</sup> mice may be more valuable for the study of E2A function during T cell development.

There are many additional applications for the E2A<sup>ER</sup> system. In addition to advantages in speed, expression level, and reversibility, this inducible system may allow analysis of E2A function in specific cell stages that have been difficult to analyze in previous E2A-deficient models. Tamoxifen treatment of E2A<sup>ER</sup> cells can allow

progression to developmental stages that are absent in E2A-deficient animals or affected due to the lack of E2A at earlier developmental stages. E2A<sup>ER</sup> mice also provide a useful tool for studying changes in E2A function with age or for analyzing E-protein function in combination with other genetic models. Given the rapid induction of E2A<sup>ER</sup> DNA binding activity upon tamoxifen treatment, we believe the E2A<sup>ER</sup> mouse model will be most valuable for identifying new E2A targets and studying the kinetics of E2A gene regulation.

## 4. E-proteins enforce thymocyte positive selection

The contents of this dissertation chapter have been slightly modified from the following publication:

Jones, M.E., and Zhuang, Y. (2007). Acquisition of a functional T cell receptor during T lymphocyte development is enforced by HEB and E2A transcription factors. *Immunity* 27, 860-870.

### 4.1 Introduction

Production of a diverse, self-tolerant compartment of  $\alpha\beta$  T cells is dependent on precise coordination of antigen receptor recombination, differentiation, and selection events during development in the thymus. The primary determinant of progression through  $\alpha\beta$  T cell development is the expression of a functional TCR. TCR expression and selection are obligatory events for the development of SP cells that will then emigrate from the thymus to establish the peripheral CD4 helper and CD8 cytotoxic T cell compartments.

The transition from DP to SP stage, directed by TCR-mediated positive selection, involves the activity of the structurally and functionally related E protein transcription factors HEB and E2A encoded by the genes *Tcf12* and *Tcf2a*, respectively (Murre, 2005). Although HEB and E2A are required and up-regulated during the early stages of T cell development, E2A activity is subsequently down-regulated as thymocytes progress through DP and SP stages (Bain et al., 1997a; Barndt et al., 1999; Engel et al., 2001; Pan et al., 2002; Taghon et al., 2006). In particular, signaling by the pre-TCR in DN thymocytes

has been shown to result in a down-regulation of E2A activity for entry to DP stage, and a similar mechanism has been proposed downstream of TCR signaling for the DP to SP transition (Engel et al., 2001; Murre, 2005). TCR signaling during a positive selection event has been suggested to reduce E2A activity by inducing expression of the E-protein inhibitor Id3 (Bain et al., 2001). *Id3* transcription can be activated downstream of ERK MAPK signaling and calcium signaling by Egr1 and NFATc1, respectively (Bain et al., 2001; Koltsova et al., 2007). In addition, E2A-deficient mice demonstrate an increase in maturation from the DP to SP stage, whereas Id3-deficient mice demonstrate a decrease in DP to SP maturation (Bain et al., 1999; Rivera et al., 2000). These findings suggest that the down-regulation of E-protein activity upon positive selection is critical to proceed past the TCR checkpoint to the SP stage of development.

Two major issues remain to be addressed. First, TCR signaling in the late DP stage triggers multiple downstream events in addition to down-regulation of E2A. Loss of E2A alone is clearly not sufficient to initiate the transition from DP to SP. It is not known whether the removal of both E2A and HEB provides the only switch or one of many parallel regulatory events leading to SP development. Second, although down-regulation of E2A can facilitate the DP to SP transition, the exact role for E-proteins during the DP stage, prior to TCR expression is not known. Here we examined E-protein function by simultaneous removal of both HEB and E2A at the DP stage. We found that the premature loss of HEB and E2A triggers development of CD8<sup>+</sup> T cells

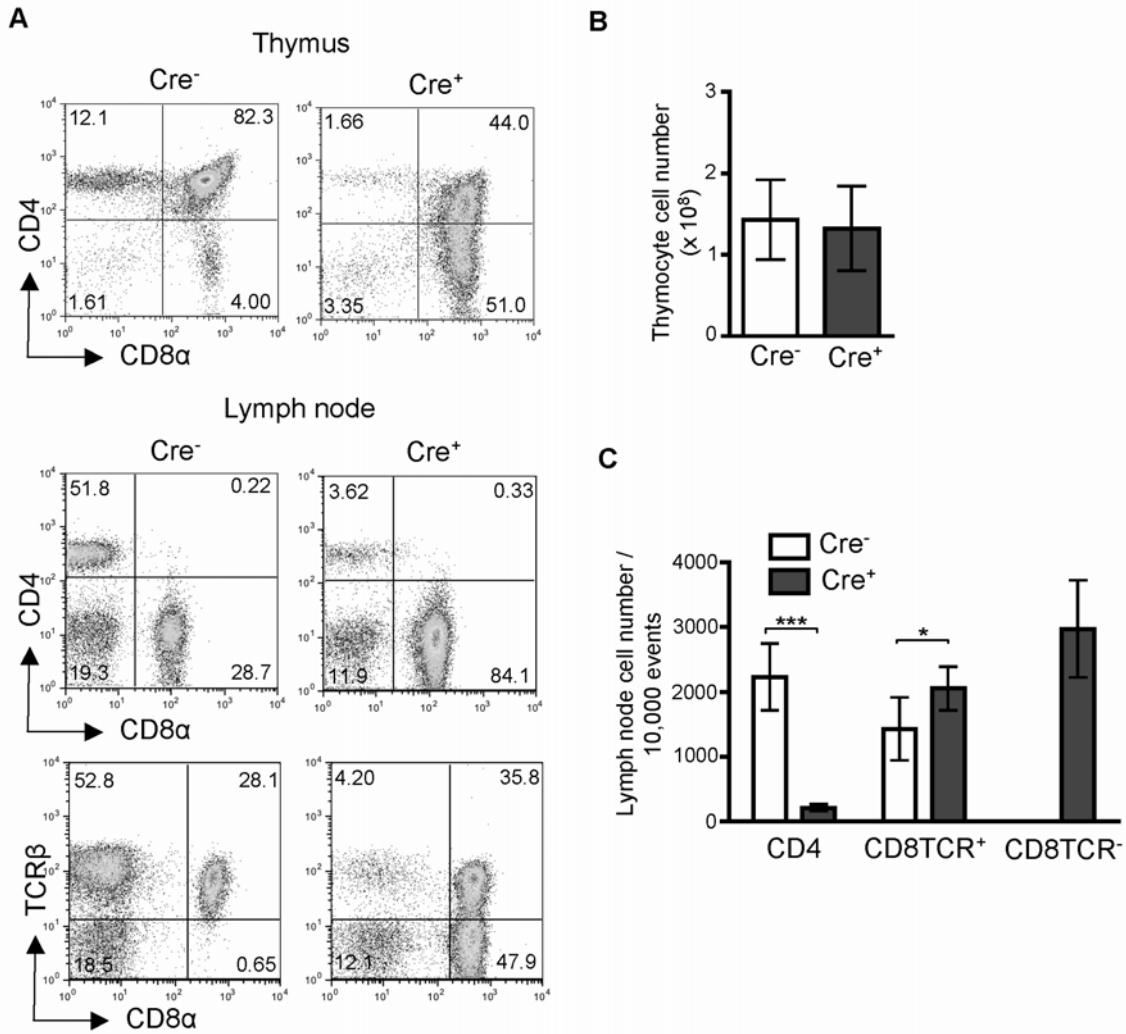
even in the absence of a TCR. Our findings identify a function for HEB and E2A in maintaining DP fate and enforcing TCR-mediated positive selection. Loss of HEB and E2A activity is not only necessary but also sufficient for development to the SP stage.

## **4.2 Results**

### **4.2.1 T cell specific deletion of E2A and HEB in DP thymocytes**

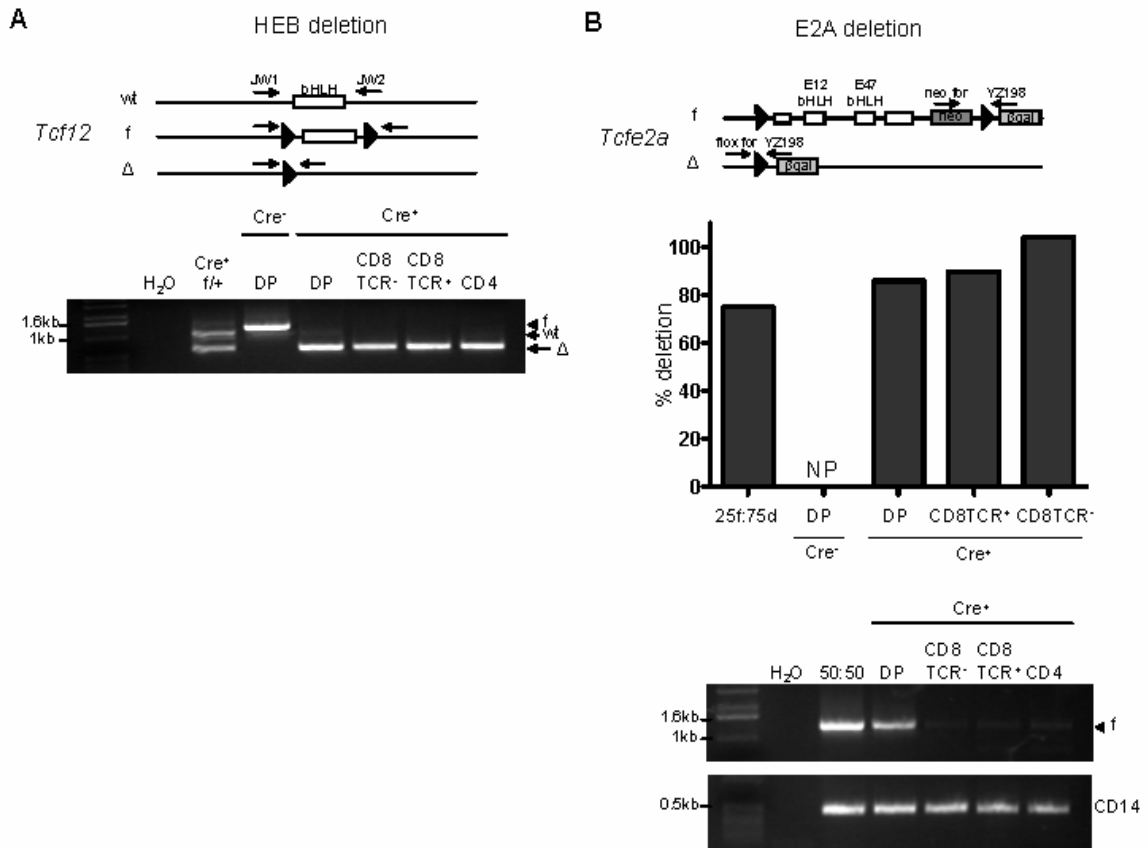
To further investigate the role in positive selection and identify additional roles for E-proteins during DP to SP development that may have been masked in previous studies, we have created a mouse model for T lineage specific deletion of both HEB and E2A in DP thymocytes. We have crossed mice carrying HEB (Wojciechowski et al., 2007) and E2A (Pan et al., 2002) conditional alleles to CD4Cre transgenic mice (Wolfer et al., 2001), and these mice will hereafter be referred to as *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice. Upon initial characterization of *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice, we observed that the CD4 and CD8 populations were markedly altered in the thymus and periphery (Figure 15). Although the overall thymic cellularity remained unchanged (Figure 15B), there was a severe reduction of CD4SP and an increase of CD8SP cells in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice compared to *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* control mice (Figure 15A). This reduction of CD4<sup>+</sup> and increase of CD8<sup>+</sup> cells was also observed in the periphery (Figure 15A,C). Even more striking, there was an abundant population of CD8<sup>+</sup> cells lacking surface TCR expression in the periphery (Figure 15A,C). Deletion analysis demonstrated that both HEB and E2A were efficiently deleted in DP and subsequent stages of development

(Figure 16). However, to determine if the CD8TCR<sup>-</sup> phenotype was dependent on deletion of all four alleles, we analyzed mice retaining either one HEB or E2A wild-type allele (Figure 17). Peripheral CD8TCR<sup>-</sup> cells were not detected in *Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* or *Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice, demonstrating that complete deletion of HEB and E2A was required for generation of this population.



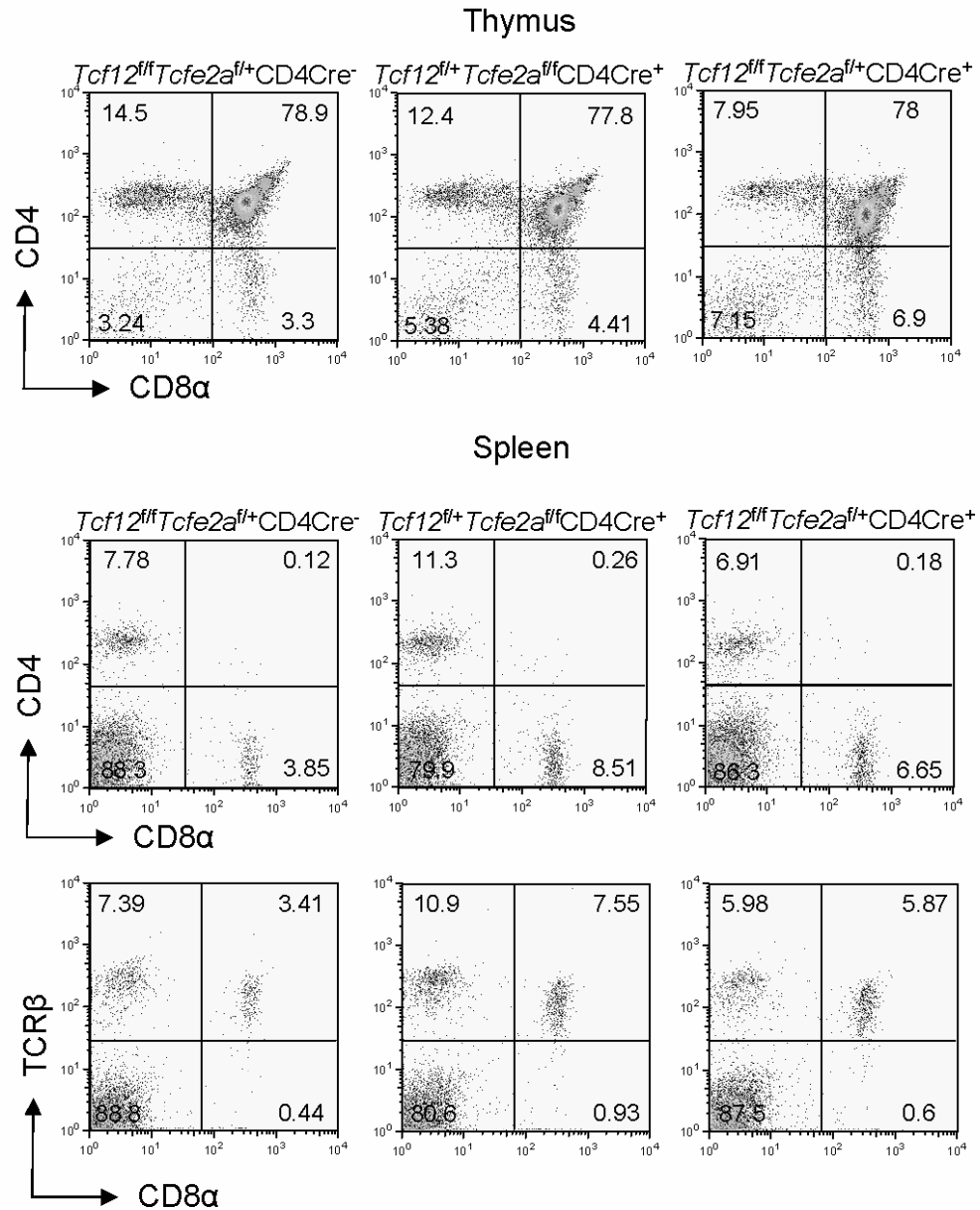
**Figure 15: T cell specific deletion of HEB and E2A generates peripheral CD8TCR<sup>-</sup> cells**

(A) Representative staining of indicated tissues from 2 month old *Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* control and *Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice for CD4, CD8α, and TCRβ. Percentages in each quadrant are displayed. (B) Cell number in the thymus of 2-3 month old mice (*n*=9). (C) Cell numbers per 10,000 events collected from lymph node (LN) of 2-7 month old mice (*Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* *n*=7, *Tcf12<sup>fl/fl</sup>Tcf2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* *n*=8). \*\*\**P*<0.001 and \**P*=0.012. Graphed results in B and C are means with error bars representing SD



**Figure 16: HEB and E2A are efficiently deleted in the DP stage of development**

Analysis of deletion efficiency in sorted populations (DP cells from thymus, CD8 and CD4 cells from LN) from *Tcf12<sup>fl/+</sup>Tcf2a<sup>fl/+</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/+</sup>Tcf2a<sup>fl/+</sup>CD4Cre<sup>-</sup>* control mice. Primer design designated in diagrams for wild-type (wt), flox (f), and deleted (Δ) alleles. (A) HEB deletion. Primers JW1 and JW2 identify f, wt, and Δ alleles. CD4 LN cells (Cre<sup>+</sup> f/+) from a *Tcf12<sup>fl/+</sup>CD4Cre<sup>+</sup>* mouse were used as a control for 50% deletion. (B) E2A deletion. Primers neo for and YZ198 detect the flox allele, and primers flox for and YZ198 detect the deleted allele. For quantitative PCR, primers flox for and YZ198 are used. A 25:75 mix of flox and deleted alleles, respectively, is used as a control for 75% deletion. Regular PCR for the flox allele only, using primers neo for and YZ198, at 40 cycles verifies qPCR results. A 50:50 mix of flox and deleted alleles is used as a control for 50% deletion. CD14 is used as a loading control. NP (no product).

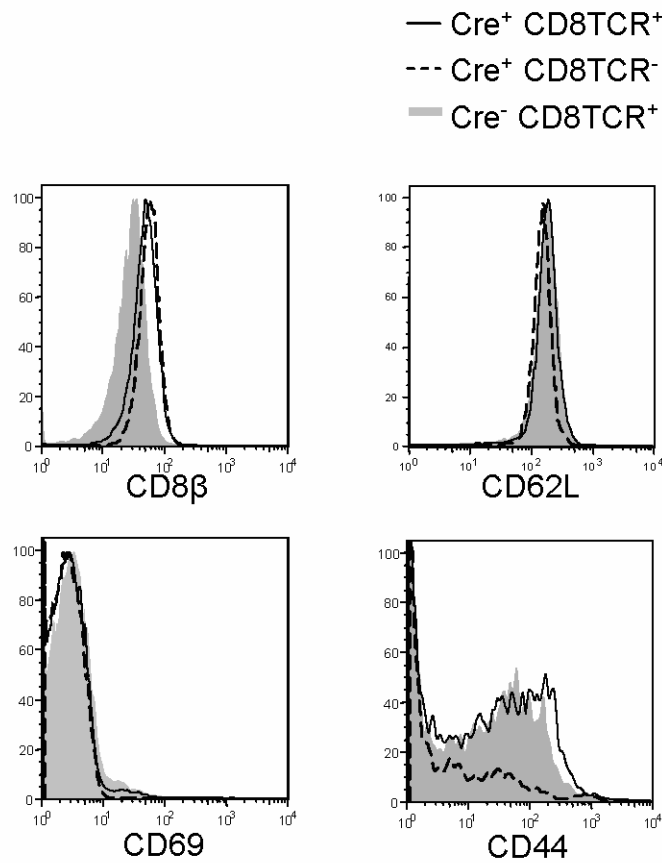


**Figure 17: CD8TCR<sup>-</sup> cells are not detected in mice retaining one HEB or E2A wild-type allele**

Representative staining of indicated tissues from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/+</sup>CD4Cre<sup>-</sup>* control, *Tcf12<sup>fl/+</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>*, and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/+</sup>CD4Cre<sup>+</sup>* mice for CD4, CD8α, and TCRβ. Percentages in each quadrant are displayed.

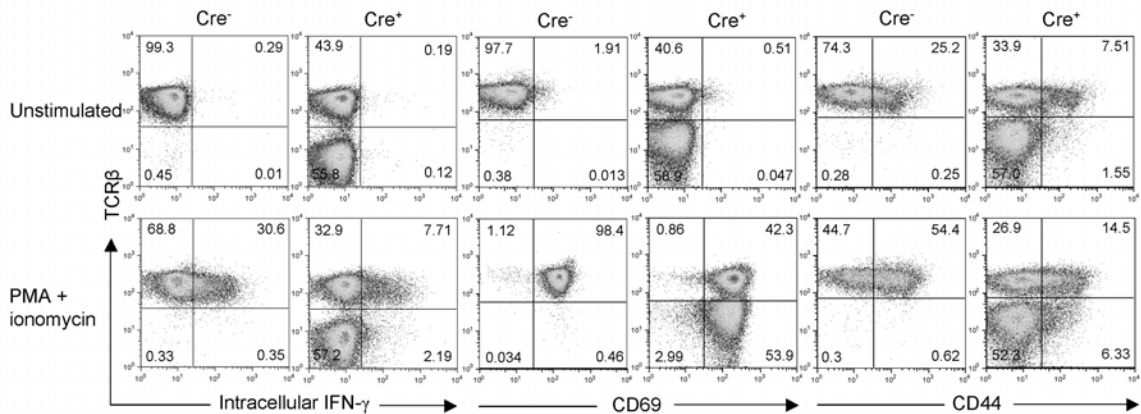
#### 4.2.2 Phenotypic characterization and functionality of CD8TCR<sup>-</sup> cells

Further analysis of the surface phenotype of the peripheral CD8TCR<sup>-</sup> cells showed that they were CD8 $\alpha$ <sup>+</sup>CD8 $\beta$ <sup>+</sup>CD62L<sup>+</sup>CD44<sup>lo</sup>CD69<sup>-</sup>TCR $\gamma\delta$ <sup>-</sup>, consistent with a mature, resting  $\alpha\beta$  T cell phenotype (Figure 18). To test if CD8TCR<sup>-</sup> cells displayed similar functionality to that of conventional CD8<sup>+</sup> T cells, we analyzed their response to stimulation. CD8TCR<sup>-</sup> cells were able to produce IFN- $\gamma$ , albeit at reduced amounts, and up-regulate expression of activation markers CD44 and CD69 upon PMA and ionomycin stimulation (Figure 19). However, a defect in homeostatic proliferation was observed in the CD8TCR<sup>-</sup> cells when transferred to lymphopenic, *Rag2*<sup>-/-</sup> recipients (Figure 20). Because these cells resembled CD8<sup>+</sup> T cells in terms of phenotype and function, but exhibited defective expansion in the periphery, they were likely generated by a constant output from the thymus.



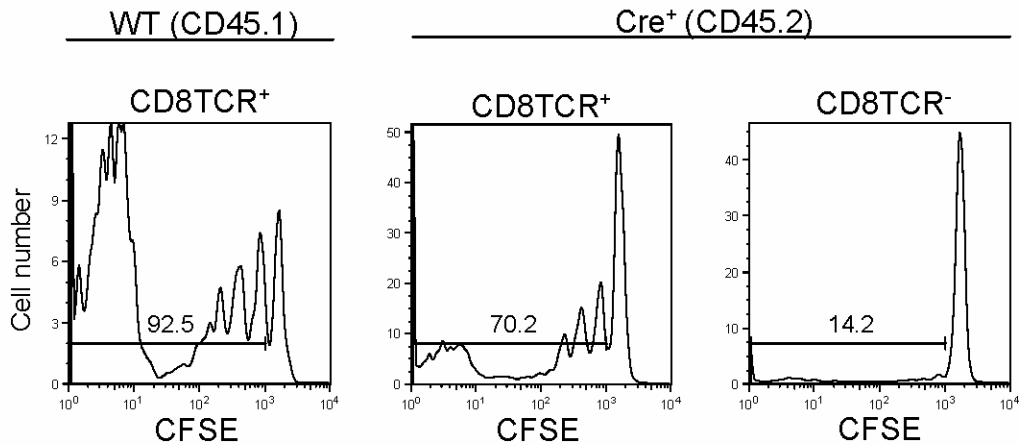
**Figure 18: Peripheral CD8TCR<sup>-</sup> cells demonstrate a mature resting T cell phenotype**

Representative staining of LN cells from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* mice. Cells are pre-gated on CD8 $\alpha$  and TCR $\beta$  surface expression. Histograms display surface expression of CD8 $\beta$ , CD69, CD62L, and CD44 for Cre<sup>+</sup> CD8TCR<sup>+</sup> cells (solid line), Cre<sup>+</sup> CD8TCR<sup>-</sup> cells (dashed line), and Cre<sup>-</sup> CD8TCR<sup>+</sup> control cells (shaded).



**Figure 19: HEB and E2A double deficient CD8TCR<sup>+</sup> and CD8TCR<sup>-</sup> cells produce IFN- $\gamma$  and up-regulate activation markers upon stimulation**

In vitro culture of LN cells isolated from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice with or without (unstimulated) PMA and ionomycin for 6hrs. Cells were analyzed by FACS analysis for intracellular IFN- $\gamma$  and surface CD69 and CD44 expression. Plots are gated on CD8<sup>+</sup> cells, and percentages in each quadrant are displayed. Data are representative of 3 independent experiments.



**Figure 20: HEB and E2A double deficient CD8TCR<sup>-</sup> cells demonstrate a defect in homeostatic proliferation when transferred to a lymphopenic host**

Total LN cells isolated from wild type (CD45.1) and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* (CD45.2) mice were mixed at a 1:3 ratio, respectively, labeled with CFSE, and intravenously injected into *Rag2<sup>-/-</sup>* deficient hosts. FACS analysis on spleen cells was performed 7 days post transfer. CFSE histograms are gated on the CD45 congenic marker, CD8 $\beta$ , and TCR $\beta$  as labeled. Plots are representative of 3 independent transfers. Percentages of cells undergoing one or more division are shown.

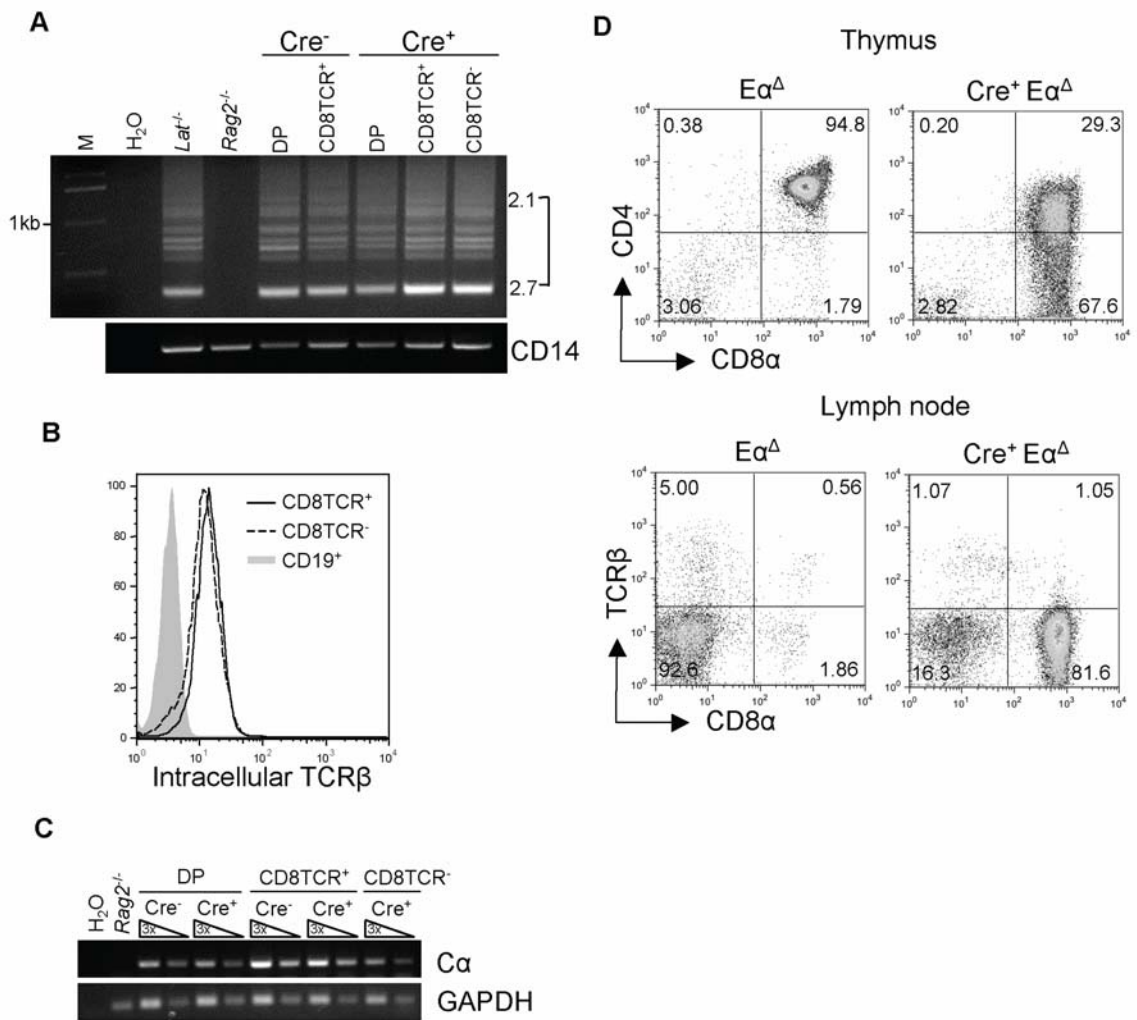
#### 4.2.3 CD8TCR<sup>-</sup> cells develop in the absence of a functional TCR

To begin investigating how and when CD8TCR<sup>-</sup> cells diverged from conventional CD8<sup>+</sup> T cell development, we first examined their TCR rearrangement status. CD8TCR<sup>-</sup> cells had undergone TCR $\beta$  V to DJ recombination and expressed intracellular TCR $\beta$  chain at a comparable amount to their CD8TCR<sup>+</sup> counterparts (Figure 21A,B). In addition, TCR $\alpha$  transcript expression was detected in the CD8TCR<sup>-</sup> cells (Figure 21C). We next performed sequencing analysis of TCR $\alpha$  V to J rearrangement by amplifying cDNA from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* CD8TCR<sup>+</sup> and CD8TCR<sup>-</sup> peripheral cells with V $\alpha$ 8

and C $\alpha$  (constant region) specific primers. Sequencing revealed that TCR $\alpha$  rearrangements were present in the CD8TCR $^-$  population, and they were non-functional, explaining why these cells lacked a functional surface TCR (Table 5). To then determine if the development of CD8TCR $^-$  cells could occur independently of TCR $\alpha$  rearrangement, we crossed our *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice onto a *Tcra* enhancer-deficient background (*E $\alpha$ <sup>Δ</sup>*) (Sleckman et al., 1997). *E $\alpha$ <sup>Δ</sup>* mice exhibit a severe block in TCR $\alpha$  recombination and accumulation of cells at the DP stage (Sleckman et al., 1997). The presence of CD8TCR $^-$  cells in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>E $\alpha$ <sup>Δ</sup>* mice showed that development of this population did not require TCR $\alpha$  rearrangement (Figure 21D).

**Figure 21: CD8TCR $^-$  cells are T cells developing in the absence of a functional TCR**

(A) TCR $\beta$  V to DJ rearrangement analysis on DNA from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* sorted thymus (DP) and LN (CD8TCR $^+$ , CD8TCR $^-$ ) populations using V $\beta$ 8 5' consensus and J $\beta$ 2.7 3' primers. Rearrangement products involving J $\beta$ 2.1-7 are shown. *Lat<sup>-/-</sup>* and *Rag2<sup>-/-</sup>* total thymocyte DNA were used as positive and negative controls, respectively. CD14 was used as a loading control. Molecular weight marker is labeled (M). (B) Intracellular TCR $\beta$  expression in specified populations from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* LN. (C) RT-PCR analysis for TCR $\alpha$  (C $\alpha$ ) expression in sorted populations from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice. *Rag2<sup>-/-</sup>* total thymocyte cDNA was used as a negative control, and GAPDH was used as a loading control. 3 fold serial dilutions are as shown. (D) Phenotype of thymus and lymph node cells from *E $\alpha$ <sup>Δ</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>E $\alpha$ <sup>Δ</sup>* mice. Percentages in each quadrant are displayed.



**Figure 21: CD8TCR<sup>-</sup> cells are T cells developing in the absence of a functional TCR**

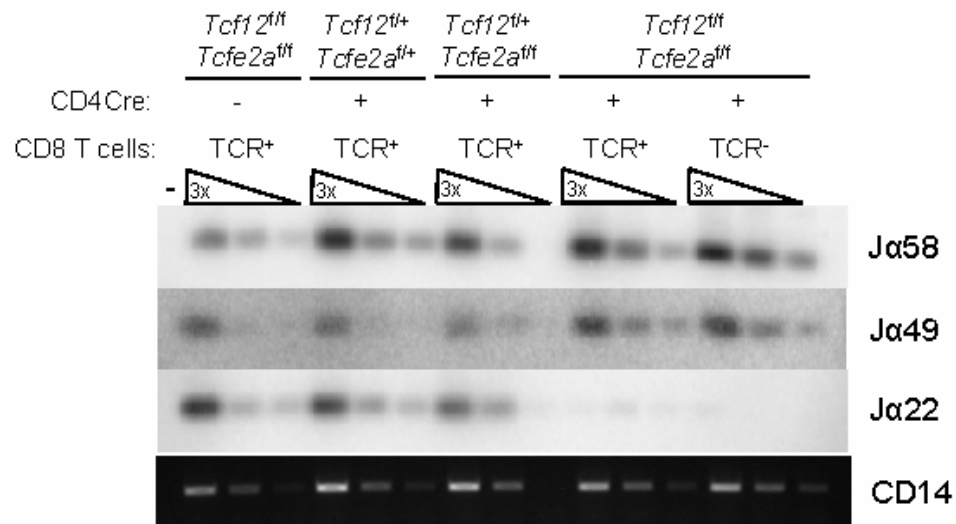
**Table 5: TCR $\alpha$  rearrangements in CD8TCR $^-$  cells are non-functional**

<b>J<math>\alpha</math></b>	<b># of clones</b>	<b># in frame</b>	<b># out of frame</b>
CD8 TCR $^+$			
57	1	1	0
52	3	3	0
47	1	0	1
42	3	3	0
<b>total</b>	<b>8</b>	<b>7</b>	<b>1</b>
CD8 TCR $^-$			
58	5	0	5
56	1	0	1
43	2	0	2
<b>total</b>	<b>8</b>	<b>0</b>	<b>8</b>

#### 4.2.4 Reduced survival of E-protein deficient DP thymocytes

The strategy used for the above sequencing of TCR $\alpha$  rearrangements also allowed for investigation of J $\alpha$  segment usage. Rearrangement in the J $\alpha$  locus proceeds in a proximal to distal (5' to 3') manner (Petrie et al., 1995; Thompson et al., 1990; Wang et al., 1998). Initial TCR $\alpha$  rearrangements use J $\alpha$  segments at the 5' end of the locus, which can be followed by secondary rearrangements using more 3' J $\alpha$  segments. In our sequencing analysis, J $\alpha$  usage in both CD8TCR $^+$  and CD8TCR $^-$  populations from *Tcf12 $^{fl/fl}$ Tcfe2a $^{fl/fl}$ CD4Cre $^+$*  mice appeared to be skewed to the 5' end of the locus (Table 5). Analysis of J $\alpha$  usage by PCR from genomic DNA of peripheral T cells also demonstrated this biased usage of the more 5' J $\alpha$  segments (Figure 22). Rearrangements using J $\alpha$ 22, the most 3' J $\alpha$  analyzed, were greatly reduced in *Tcf12 $^{fl/fl}$ Tcfe2a $^{fl/fl}$ CD4Cre $^+$*  CD8TCR $^+$  and

CD8TCR<sup>-</sup> T cells compared to *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and heterozygous *Tcf12<sup>fl/+</sup>Tcfe2a<sup>fl/+</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/+</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* controls, whereas rearrangements using the more 5' J $\alpha$ 58 and J $\alpha$ 49 segments were equally if not more abundant compared to controls. These results were reminiscent of the ROR $\gamma$ -deficient mice that demonstrate a defect in 3' J $\alpha$  usage due to a survival defect limiting the lifespan of DP thymocytes (Guo et al., 2002; Kurebayashi et al., 2000; Sun et al., 2000). Because E2A has been shown to regulate the isoform ROR $\gamma$ t in DP thymocytes (Xi et al., 2006), we next analyzed ROR $\gamma$ t expression. As suspected, ROR $\gamma$ t expression was decreased in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells (Figure 23A). In addition, *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP thymocytes demonstrated reduced ex vivo survival when cultured in media alone compared to wild-type, *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>*, and *E $\alpha$  <sup>$\Delta$</sup>*  controls (Figure 23B). Together, the J $\alpha$  usage, decrease in ROR $\gamma$ t expression, and reduced ex vivo survival suggested that *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* thymocytes too have a shortened time in the DP stage.

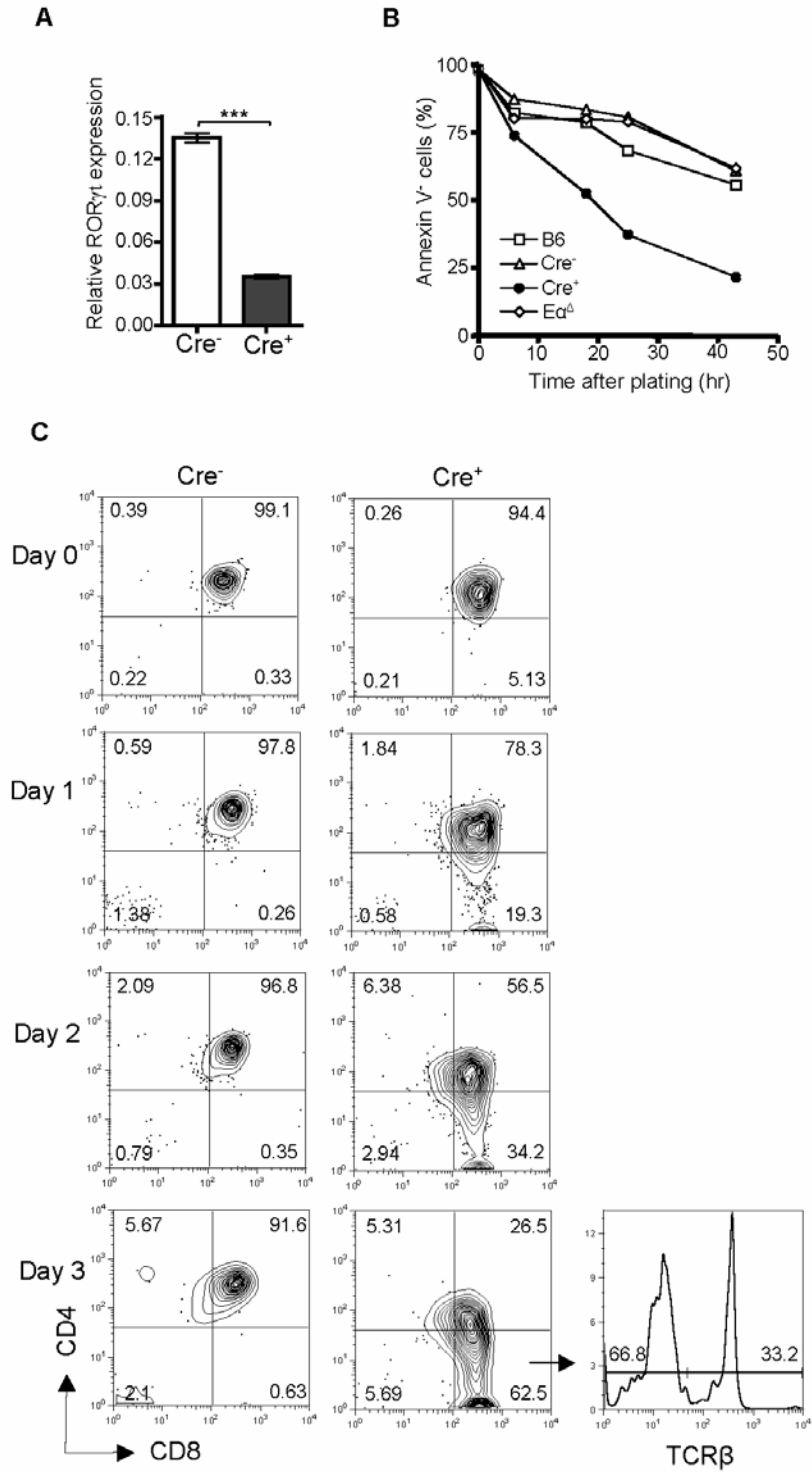


**Figure 22: *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* T cells demonstrate a skewing of Jα usage to the 5' end of the locus**

Analysis of Jα 58, 49, and 22 usage in TCRα rearrangements from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>*, *Tcf12<sup>fl/+</sup>Tcfe2a<sup>fl/+</sup>CD4Cre<sup>+</sup>*, *Tcf12<sup>fl/+</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>*, and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* sorted peripheral CD8 T cells. PCR and Southern blot analysis of Vα8 to Jα rearrangement. CD14 was used as a DNA loading control. Water was used as a negative control (-). Results are from one mouse per genotype. Jα58, 49, and 22 are located 5' to 3' within the Jα locus.

**Figure 23: *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP thymocytes survive poorly, but differentiate to SP cells efficiently in culture**

**(A) Quantitative RT-PCR analysis of ROR $\gamma$ t expression in sorted *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells. Samples were normalized to the expression of GAPDH. Data are from duplicates of two independent experiments (n=4). \*\*\*P<0.001. Graphed results are means with error bars representing SEM. (B and C) Ex vivo culture analysis of sorted DP thymocytes from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice. (B) DP cells were plated in media alone and analyzed for Annexin V expression by FACS at 0, 6, 18, 25, and 43hrs after plating. Wild-type (B6) and E $\alpha^A$  DP cells were used as additional controls. Data are representative of two independent experiments. (C) DP cells were plated on a layer of total thymic stromal cells (Day 0) and analyzed by FACS analysis for CD4, CD8, and TCR $\beta$  expression on Day 1-3. Percentages in each quadrant are displayed. TCR $\beta$  expression within the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* CD8SP gate is shown for Day 3. Data are representative of 3 independent experiments.**



**Figure 23: *Tcf12<sup>fl/t</sup>Tcfe2a<sup>fl/t</sup>CD4Cre<sup>+</sup>* DP thymocytes survive poorly, but differentiate to SP cells efficiently in culture**

## 4.2.5 Loss of E2A and HEB initiates CD8 T cell development

### 4.2.5.1 E-protein deficient DP thymocytes efficiently differentiate into SP cells

Even though the data suggested that CD8TCR<sup>-</sup> cells developed from DP thymocytes, it still remained possible that these cells, being CD8<sup>+</sup>, could develop directly from the earlier CD8<sup>+</sup> ISP stage. ISP cells represent a transitional stage from DN to DP development when cells first up-regulate CD8 prior to CD4. To therefore verify the DP stage as the developmental source of our CD8TCR<sup>-</sup> cells, we cultured DP thymocytes *ex vivo* on a thymic stromal layer. DP thymocytes from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice not only gave rise to both CD8TCR<sup>+</sup> and CD8TCR<sup>-</sup> SP cells, but did so very efficiently compared to the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* control cells (Figure 23C).

*Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells appeared to have been rescued from an enhanced cell death phenotype (Figure 23B) because the loss of HEB and E2A also triggered maturation of DP cells to the SP stage. It is this latter event that led to the development of CD8TCR<sup>-</sup> cells.

We then wanted to analyze the rate of CD8SP cell production *in vivo*.

*Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and wild-type (B6) mice were injected with BrdU for 4 and 24hr hour pulse analysis. Percent labeling in DN, DP and CD8SP populations did not demonstrate a dramatic difference between *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and control mice at either time point (Figure 24). However, *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice did display an increase in BrdU<sup>+</sup> CD8SP cell numbers at 24hrs. Because cell cycle analysis by Hoechst staining indicated *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* CD8SP cells were not proliferating (data not

shown), the increase in BrdU<sup>+</sup> CD8SP cell number could be due to more DP cells giving rise to CD8SP, an increased rate of DP to CD8SP differentiation, or a combination of both.

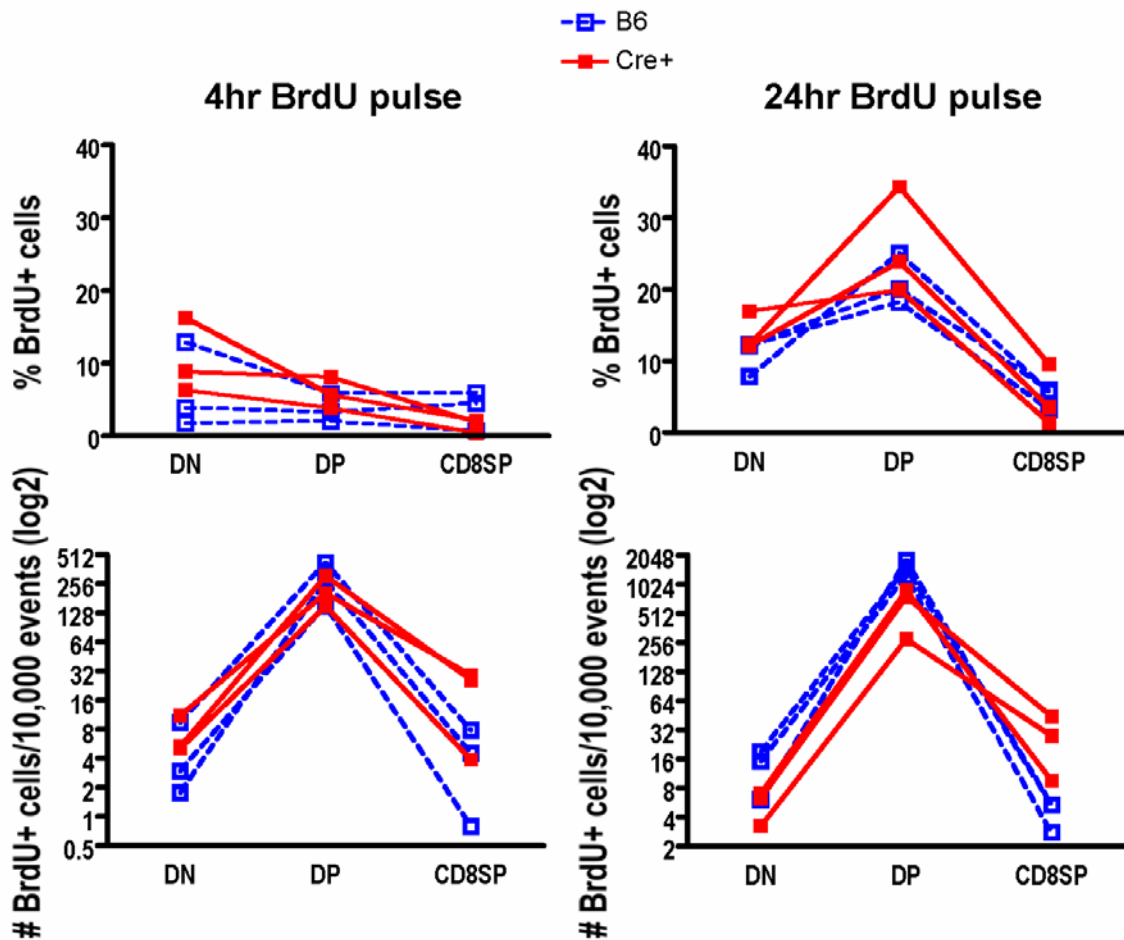


Figure 24: *In vivo* BrdU labeling demonstrates generation of more CD8SP cells in  $Tcf12^{fl/fl}Tcfe2a^{fl/fl}CD4Cre^+$  mice

Wild-type (B6) and  $Tcf12^{fl/fl}Tcfe2a^{fl/fl}CD4Cre^+$  mice were IP injected with BrdU and analyzed 4 or 24hrs after injection. Thymocytes were stained for CD4, CD8, and TCR $\beta$  surface expression, and then processed to analyze BrdU labeling. Graphs display percent (top panel) and cell number (lower panel) of BrdU+ cells within each pre-gated population: DN (CD4<sup>-</sup>CD8<sup>-</sup>), DP (CD4<sup>+</sup>CD8<sup>+</sup>), and CD8SP (CD4<sup>-</sup>CD8<sup>+</sup>TCR $\beta$ <sup>+</sup>).

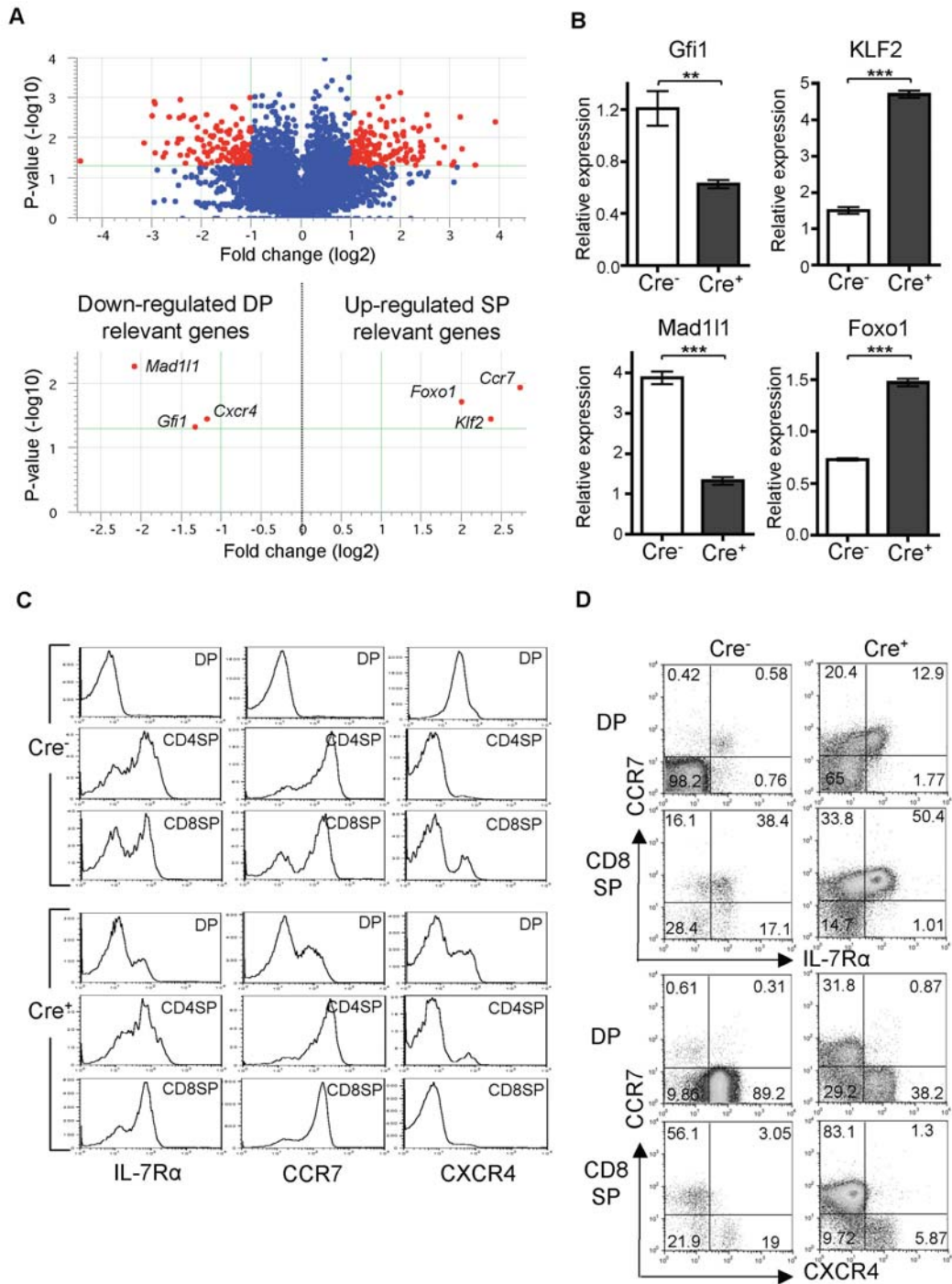
#### 4.2.5.2 E-protein deficient DP thymocytes reveal SP-like gene expression

The above data suggested that HEB and E2A are required to maintain DP fate and prevent premature differentiation to the SP stage. To get a comprehensive view of HEB and E2A mediated gene expression during the DP stage, we performed microarray analysis using sorted DP cells. The results yielded a group of 285 genes that were either up-regulated or down-regulated greater than 2 fold in the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP population compared to *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* control DP cells (Figure 25A and 3 fold change listed in Tables 6-7). Within these groups an interesting trend emerged. Genes known to be highly expressed at the DP stage were down-regulated within the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP population, whereas genes known to be up-regulated at the SP stage were already being up-regulated. For example, *Gfi1* and *Mad111*, which are two genes whose expression has been shown to drop dramatically upon differentiation to SP stage (Rudolph et al., 2001; Yucel et al., 2003), were both down-regulated in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells. In contrast, *Foxo1*, which is most highly expressed in positively selected DP cells and SP cells (Leenders et al., 2000), was up-regulated in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP thymocytes. Of these, *Gfi1* is of particular interest because *Gfi1<sup>-/-</sup>* mice also demonstrate an enhanced development of CD8SP cells, however shown in only a TCR-dependent manner (Yucel et al., 2003). Altered expression of *Gfi1*, *Mad111*, and *Foxo1* in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP thymocytes was also confirmed by quantitative RT-PCR (Figure 25B).

The DP microarray analysis also identified genes that are relevant to thymocyte migration. *Klf2*, encoding a transcription factor critical for activating expression of S1P<sub>1</sub> during SP maturation to allow thymic egress of mature T cells (Carlson et al., 2006), was up-regulated in our *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells. KLF2 expression was also verified by quantitative RT-PCR analysis (Figure 25B). As discussed in Chapter 1, the chemokine receptor CXCR4 is expressed in early stages of T cell development for cortical retention within the thymus (Plotkin et al., 2003), and it is then down-regulated upon positive selection. Concurrently, the chemokine receptor CCR7 is up-regulated at this time to induce migration of selected SP thymocytes from the cortex to the medulla, where they will then undergo negative selection (Kurobe et al., 2006). We found that *Cxcr4* and *Ccr7* were down-regulated and up-regulated, respectively in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP thymocytes. In accordance with these microarray results, the surface expression of IL-7R $\alpha$  and CCR7, which are both up-regulated from DP to SP, were already up-regulated in a fraction of *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells at the level of that in SP stage cells (Figure 25C). In addition, surface expression of CXCR4 was predominantly down-regulated in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells. To ensure that these were coordinated events in individual cells, we analyzed staining of CCR7 and IL-7R $\alpha$  or CXCR4 together. *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells that had up-regulated CCR7 expression had concurrently up-regulated IL-7R $\alpha$  and down-regulated CXCR4 in a manner similar to that of the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* SP cells (Figure 25D).

**Figure 25: Loss of HEB and E2A initiates CD8 T cell maturation and thymic egress in the absence of a TCR-mediated positive selection signal**

**(A)** Volcano plot from microarray data comparing gene expression in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* DP thymocytes. Changes in gene expression are shown as a ratio of *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* / *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* cells. Upper plot shows the 15,730 genes remaining after quality filtering, with the 285 genes with greater than 2 fold change and *t*-test *p*-value  $\leq 0.05$  in red. Lower plot highlights a few genes of interest. **(B)** Quantitative RT-PCR analysis of *Gfi1*, *Mad111*, *KLF2*, and *Foxo1* expression in sorted *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells. Samples were normalized to the expression of GAPDH. Data are from duplicates of two independent experiments (n=4). \*\*\**P*<0.001 and \*\**P*=0.0054. Graphed results are means with error bars representing SEM. **(C and D)** FACS analysis of IL-7R $\alpha$ , CCR7, and CXCR4 expression in DP compared to SP stage in thymus from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice. Cells are pre-gated on CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>+</sup>CD8<sup>-</sup> (CD4SP), and CD4<sup>-</sup>CD8<sup>+</sup> (CD8SP) populations. **(C)** Histograms display IL-7R $\alpha$ , CCR7, and CXCR4 expression in designated populations from individual stainings. **(D)** FACS plots demonstrate coordinated expression of CCR7 and IL-7R $\alpha$  or CXCR4 in designated populations with percentages in each quadrant displayed. Data are representative of two independent experiments.



**Figure 25: Loss of HEB and E2A initiates CD8 T cell maturation and thymic egress in the absence of a TCR-mediated positive selection signal**

**Table 6: List of microarray identified genes down-regulated greater than 3 fold in *Tcf12<sup>flk</sup>Tcfe2a<sup>flk</sup>CD4Cre<sup>+</sup>* DP cells with a p-value  $\leq 0.05$**

Cre <sup>+</sup> /Cre <sup>-</sup> fold change	Ensembl	Unigene	Common Name
0.0459	Smoc1	Mm.273295	SRG; AI848508; 2600002F22Rik
0.125		Mm.186434	C43; Mf3; Twh; Fkh5; Foxb1a; Foxb1b; Hfh-e5.1; MGC130499
0.128	Prodh		
0.13		Mm.48648	KIAA0523; MGC55022; mKIAA0523; RP23-223C15.3
0.131	Ppfibp2	Mm.2817	Cclp1; MGC117695
0.145	Olig3	Mm.156946	Bhlhb7
0.149	Rragd		
0.158	Cbfa2t3h		
0.18	ldb3		
0.182	Car2	Mm.1186	CAII; Car-2; Ltw-5; Lvtw-5; AI131712
0.182	Tec	Mm.319581	Tsc2
0.186	Egr2	Mm.290421	Egr-2; Zfp-6; Krox20; NGF1-B; Zfp-25; Krox-20
0.187		Mm.330745	kr; Krml; Krml1; Kreisler
0.187		Mm.28456	Pro1; Pro-1; Ym24d07
0.194	Pip5k1a		
0.198		Mm.314618	Zak; MLTK; AV006891; MLTKbeta; MLTKalpha
0.205	Pip5k1a		
0.211	Ephx2	Mm.15295	Eph2; AW106936
0.216	Lass4		
0.223	Irf6		
0.225	Plxdc1	Mm.39617	Tem7; AI848450; MGC130377; 2410003I07Rik
0.235	Nab2	Mm.336898	AI451907
0.236	Mad111	Mm.27250	Mad1; AI173502; AW550425
0.236	Dusp6		
0.237	Zdhhc14		
0.247	Arl6ip2		
0.25	Egr1		egr; TIS8; Zenk; Egr-1; NGFIA; Zfp-6; ETR103; Krox-1; Krox24; NGF1-A; NGFI-A; Zif268; Krox-24; A530045N19Rik
0.26	Hrbl	Mm.260869	RABR; MGC7148; MGC61203; A630095P14Rik
0.263	D10Bwg09 40e	Mm.297971	BC013565; KIAA1357; mKIAA1357; A730096F01; D10Bwg0940e
0.269		Mm.38269	LOC666794
0.28		Mm.227912	opt; Ip3r; P400; Pcp1; IP3R1; Pcp-1; D6Pas2; InsP3R; Itpr-1
0.282	Fbp1	Mm.423078	Fbp2; Fbp-2; FBPase 1
0.283	H2-D1		Q10; Qa10; H-2Q10
0.293	Spg3a		
0.293	Ets2	Mm.290207	Ets-2; AU022856
0.308	Cdkl1		
0.313	Tle6	Mm.206764	Grg6; 1810057E06Rik
0.316	Slc35d1		
0.331	Rapgef3	Mm.24028	Epac; Epac1; MGC19192; 2310016P22Rik; 9330170P05Rik
0.332	Socs3	Mm.3468	CIS3; Cish3; EF-10; SSI-3; SOCS-3

**Table 7: List of microarray identified genes up-regulated greater than 3 fold in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells with a p-value  $\leq 0.05$**

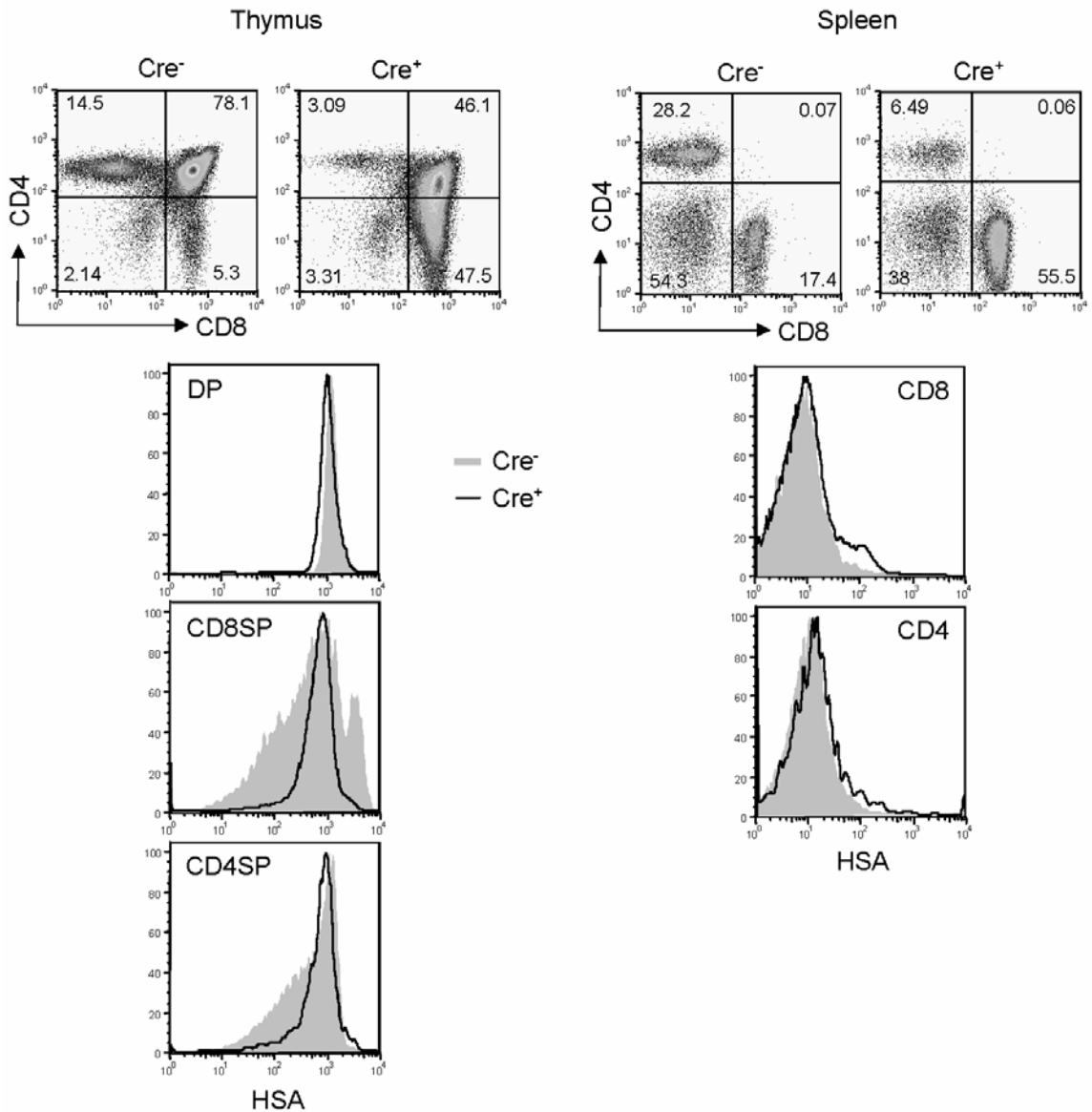
Cre <sup>+</sup> /Cre <sup>-</sup> fold change	Ensembl	Unigene	Common Name
15.13	Ctla2b		
11.4	Rapgef4	Mm.196153	Epac2; KIAA4040; mKIAA4040; 1300003D15Rik; 5730402K07Rik
9.494	Sgne1		
8.523	Tesc		
8.285	Elovl6	Mm.314113	FAE; LCE; C77826; MGC107467
7.35	Ctla2b	Mm.30144	Epb4.1l2
6.854		Mm.21814	AU018597; 6720474K14Rik; 9430079M16Rik
6.626	Ccr7	Mm.2932	EBI1; CD197; Ebi1h; Cdw197; Cmkbr7
5.917	Q8K244		
5.754		Mm.333048	Ian1; IMAP4; mIAN1; AU019574; MGC11734; E430007K16Rik
5.499	Cd53		
5.448	Pde3b	Mm.430730	AI847709
5.39		Mm.35548	Clint1
5.323	Lix1	Mm.268018	5730466L18Rik
5.296	Ssb4		
5.296	Q9CZZ7		
5.137	Piwil2	Mm.85253	mili; Piwil1l
5.131	Klf2	Mm.26938	Lklf
4.845	Dncl2b	Mm.23114	Dynlrb2
4.789	Mpra		
4.754	Gm2a	Mm.287807	AA408702; AW215435
4.621	Q9CZY7		
4.392		Mm.31817	EKN1; 1700010I24Rik
4.373	Eng	Mm.225297	CD105; AI528660; S-endoglin
4.332	Cnn2	Mm.157770	AA408047; AI324678
4.321	Ank2	Mm.220242	Ank-2; AI835472; AW491075; Ankyrin-2; Ankyrin-B; ankyrin B; ankyrin>B<
4.284	Add3	Mm.426080	R75380; AI463285
4.264	Rasa3		
4.2	Ggtla1		
4.174	Pscd4		
4.128	Gng2		
4.012	Slc26a11		
3.991	Foxo1	Mm.29891	Afxh; FKHR; Fkhr1; Foxo1a; AI876417
3.929	Pdlim1		
3.846	Q8BJ19		AI225904; 1700108N18Rik; E430013J17Rik
3.824	Scotin	Mm.196533	Scotin; 2310008D10Rik; 6430628I05Rik
3.772	Rttn		
3.766	Gig1		Gig1; C030026M03; C030034L19Rik
3.752		Mm.4065	Eeig1; AI426465; MGC38572
3.658	Rps6ka1	Mm.301827	rsk; Rsk1; p90rsk
3.619	Cst7	Mm.12965	Cst3

**Table 7 continued**

Cre <sup>+</sup> /Cre <sup>-</sup> fold change	Ensembl	Unigene	Common Name
3.582	Chd3	Mm.178246	Chd7; Prp7; Prp9-1; AF020312; MGC40857; 2600010P09Rik
3.572	Rassf3	Mm.41265	AW212023; AW322379
3.506	Zfpn1a4		
3.413	Ian3		
3.382	Itgb7		
3.305	Ly6c		
3.288	Ramp2		
3.254		Mm.393405	Clp; 1810074P22Rik; 2010004C08Rik
3.236	CNC2_M OUSE		
3.204	CATW_M OUSE		
3.11	Ms4a6d	Mm.290390	AI447446
3.067	Chd3		
3.064	Hexb	Mm.27816	Hbb-b1
3.01	Tmsb4x		

These SP-like changes in chemokine receptor expression at the DP stage in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice together with the decrease in ROR $\gamma$ t expression (Figure 23A) and skewed J $\alpha$  usage (Table 5 and Figure 22) were consistent with a shorter DP lifespan. To further investigate the dwell time of cells in the thymus we chose to analyze HSA (CD24) expression. HSA is a useful marker for T cell maturation but is not functionally critical for thymocyte development (Nielsen et al., 1997). HSA is highly expressed on ISP cells, slightly lower on DP cells, begins to be further down-regulated on SP cells, and is low or absent on mature peripheral T cells. The CD4SP and CD8SP thymocytes in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice demonstrated a DP-like staining for HSA, suggesting SP cells had not yet had time to begin down-regulating HSA expression

(Figure 26). However, the HSA expression in peripheral T cells from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice was similar to that of the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* control cells. This indicated that *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* T cells did down-regulate HSA by the time they reached the periphery. Together, the microarray and surface expression analysis showed that DP cells were prematurely acquiring an SP phenotype upon deletion of HEB and E2A.

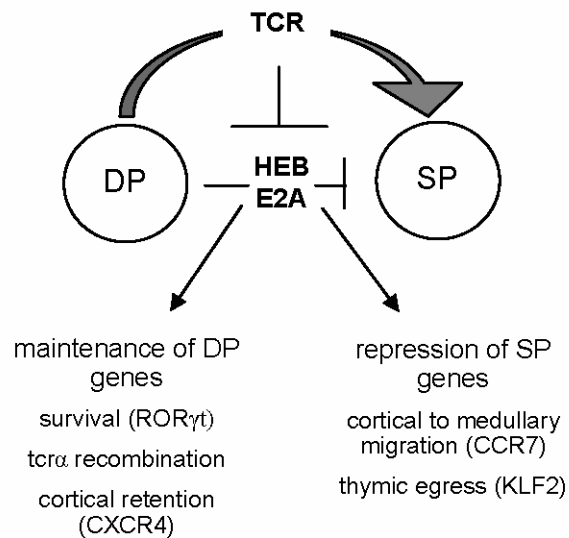


**Figure 26: *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* SP thymocytes have not yet down-regulated HSA expression to the levels detected in the periphery**

Staining of indicated tissues from *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* (shaded in histogram) and *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* (line in histogram) mice for expression of CD4, CD8, and HSA (clone M1/69). Percentages in each quadrant are displayed for CD4 vs CD8 plots. Histograms display HSA expression in gated populations from above plots: DP (CD4<sup>+</sup>CD8<sup>+</sup>), CD8SP/CD8 (CD4<sup>-</sup>CD8<sup>+</sup>), CD4SP/CD4 (CD4<sup>+</sup>CD8<sup>-</sup>).

### 4.3 Discussion

The TCR-independent maturation of *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* DP cells exhibited various aspects of CD8SP cell development, including the silencing of DP specific genes, maturation to a CD8 single positive phenotype, and activation of factors critical for migration and thymic egress. These findings have demonstrated that HEB and E2A are critical at the DP stage to block further development until a proper TCR-mediated positive selection signal is received (Figure 27). Premature withdrawal of HEB and E2A prior to this signal was sufficient to activate the developmental program for CD8 lineage, whether the cell had produced a functional TCR or not. We propose that HEB and E2A function as gatekeepers for a default pathway from DP to CD8SP stage.



**Figure 27: E-proteins function as the gatekeepers regulating the DP to SP transition**  
**A model for the role of HEB and E2A during the DP stage of T cell development.**

The *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre* mouse model will now allow us to further elucidate the downstream transcriptional network directing this pivotal differentiation step from DP to SP. It appears that E-proteins are regulating, perhaps directly, two sets of genes. HEB and E2A are required to both maintain DP gene expression and prevent SP gene activation. One candidate gene from our microarray data mentioned above is *Gfi1*, encoding a transcriptional repressor that may act with or downstream of E-proteins to suppress SP genes at the DP stage. *Gfi1* was also identified in a microarray study by Schwartz et al. as a gene suggested to be directly up-regulated by E2A (Schwartz et al., 2006). In addition, *Foxo1* was identified as an E2A target in this microarray analysis and was suggested to be directly repressed by E2A (Schwartz et al., 2006). This finding also concurs with our microarray data, indicating that *Foxo1* may be an E2A-repressed gene at DP stage that is activated upon differentiation to SP stage. These targets and others identified suggest a network of transcription factors functioning downstream of E-proteins to orchestrate proper DP to SP development.

Our observation that *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* T cells developed primarily into CD8 lineage but not CD4 suggests a role for E-proteins in lineage choice. It has been suggested that CD8 development is a default pathway, and an additional or prolonged signal (as suggested by the model in Figure 2) is required for activation of Th-POK to direct cells to the CD4 lineage (Aliahmad and Kaye, 2006; Kappes and He, 2006). The loss of HEB and E2A in our system initiated premature DP to CD8SP development.

Although HSA staining of *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* thymocytes suggests an increased maturation rate, whether or not this DP to SP transition was accelerated once initiated remains to be determined. If the development to SP stage was accelerated upon deletion of HEB and E2A in our model, it is therefore possible that the cells did not have enough time for CD4 lineage instruction. Alternatively, HEB and E2A may be required specifically for CD4 development or for suppression of CD8 development. Analysis of Th-POK expression in *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* versus *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>-</sup>* CD4SP cells demonstrated that HEB and E2A are not required for maintenance of Th-POK expression (data not shown). However, this finding does not rule out a role for E-proteins upstream of initiation of Th-POK expression.

One consideration for why the loss of E-protein activity favors CD8 over CD4 is the role of E-proteins in regulating CD4 expression. Previous studies have suggested the CD4 enhancer as a target of E-proteins during thymocyte development (Sawada and Littman, 1993). The presence of HEB and E2A deficient CD4 T cells in our *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice, albeit at greatly reduced numbers, indicates that HEB and E2A are not required for CD4 expression in mature T cells. However, it still remains possible that loss of CD4 expression, or a CD4 lineage specific gene, at DP stage upon deletion of HEB and E2A could contribute to the development of only CD8<sup>+</sup> T cells.

If the loss of HEB and E2A triggers development of CD8<sup>+</sup> T cells, why did we see this small population of peripheral CD4<sup>+</sup> T cells in the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice?

These peripheral CD4<sup>+</sup> T cells were HEB and E2A deficient, as demonstrated by deletion analysis. Because all of these CD4<sup>+</sup> T cells were TCR<sup>+</sup>, they likely originated from a small number of MHC II restricted DP cells receiving a positive selection signal prior to complete deletion of HEB and E2A. These CD4<sup>+</sup> T cells were capable of survival and homeostatic proliferation upon transfer (data not shown), so it is expected that the peripheral CD4<sup>+</sup> T cell population results from an accumulation of these rare events. It also remained possible that most of the CD4<sup>+</sup> T cells in the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice were negatively selected and those that survived were CD4<sup>+</sup> T cells with the lowest binding affinity for MHC-peptide. A role for E-proteins in negative selection is also currently being investigated.

Transcriptional regulation by E-proteins may provide a means to coordinate DP to SP differentiation, selection, and CD4 versus CD8 lineage commitment. Our results have shown that HEB and E2A are critical determinants of the DP to SP transition by enforcing the requirement for TCR-mediated positive selection. Our results also suggest that E-proteins may serve as key regulators during CD4 versus CD8 lineage choice. Positive selection and lineage choice are suggested to be tightly linked processes, however, as discussed in Chapter 1, the regulation of each remains controversial. Future analysis of the *Tcf12<sup>fl/fl</sup>Tcfe2a<sup>fl/fl</sup>CD4Cre<sup>+</sup>* mice in predefined selective backgrounds may help to distinguish the regulatory pathways driving each of these events.

## 5. Conclusions and future directions

The studies presented here utilize genetic models allowing the manipulation of E-protein function or expression. Mouse models often provide the most convincing evidence of gene function. We believe the E2A<sup>ER</sup> system offers benefits beyond previous E2A mouse models by allowing rapid and reversible regulation of E2A protein expressed from the endogenous locus in primary cells. The Tcf12<sup>f/f</sup>Tcfe2a<sup>f/f</sup>CD4Cre mouse model revealed novel functions for E-proteins at the DP stage of thymocyte development by allowing lineage and stage specific deletion of both HEB and E2A (Jones and Zhuang, 2007). These systems have led to new findings and provide direction for future studies to further dissect the roles of E-proteins during lymphocyte development.

The E2A<sup>ER</sup> system is not limited to studies in lymphocytes and may be useful to other areas of research since E2A displays an extensive range of functions across multiple developmental programs. In addition, because E2A dosage and cell context are important determinants of the outcome of E2A function, E2A<sup>ER</sup> mice provide an attractive system to regulate E2A protein function under physiological conditions. However, due to the inefficiency of *in vivo* tamoxifen treatment, many studies may be limited to *ex vivo* analysis. Even so, we anticipate this system will help clarify previously identified E2A functions in addition to identifying novel functions. Rescue of B cell development upon tamoxifen treatment of E2A<sup>ER</sup> pre-proB cells has already suggested

the ability to separate early and late E2A-regulated events for B lineage commitment. Identification of novel target genes and the kinetics of their regulation will likely enhance our understanding of complex transcriptional networks driven by E2A.

Deletion of HEB and E2A in DP thymocytes identified a critical function for E-proteins in positive selection, but also suggested that E-proteins play additional roles during this stage of development. There were two obvious T cell phenotypes upon initial analysis of  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+$  mice: the presence of a TCR<sup>-</sup> T cell population and the disrupted CD4:CD8 ratio. We determined that the TCR<sup>-</sup> T cell population resulted from a requirement of E-proteins at the DP stage to prevent further development until a TCR-mediated positive selection signal is received. The fact that all of the TCR<sup>-</sup> T cells are CD8 positive fits well with the current model for lineage commitment (Figure 2). DP cells that lack a functional TCR will not have the persistent signal to trigger CD4 lineage development. However, why then are most of the TCR<sup>+</sup> cells also CD8 lineage cells? Some more recent data we have collected suggests that E-proteins are required for the development of CD4 lineage cells. First, analysis of  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+OTII^+$  mice, which express an MHC II restricted TCR transgene (Barnden et al., 1998), demonstrated that MHC II restricted cells can be redirected to the CD8 lineage upon deletion of HEB and E2A (data not shown). In addition, crossing  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre$  mice onto a Bim deficient background demonstrated no significant rescue of the CD4 population, suggesting that negative selection is not

responsible for the CD4 lineage defect (data not shown). Therefore, we are now pursuing a role for E-proteins in CD4 versus CD8 lineage choice.

As mentioned in Chapter 4, there are possibly multiple functions for E-proteins in CD4 lineage instruction. Here I will further discuss two possible scenarios. First, HEB and E2A could be directly required upstream of a critical CD4 lineage factor, for example CD4, Gata3, or Th-POK. E-proteins have been previously implicated in other cell systems to regulate CD4 and Gata3 expression, and E-box sites can be found in Th-POK regulatory regions (He et al., 2008; Sawada and Littman, 1993; Schwartz et al., 2006; Xu and Kee, 2007). A direct requirement for E-proteins in CD4 lineage development would suggest that either positive selection does not result in a complete loss of E-protein function or E-proteins promote CD4 development prior to their complete down-regulation. Perhaps E-protein activity must drop below a certain threshold for DP cells to progress to the SP stage. The low level of remaining E-protein activity would then be required to activate CD4 lineage genes or repress CD8 lineage factors. The second possibility is that the deletion of E-proteins at the DP stage in  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre$  mice may result in development of CD8 lineage cells because there is not enough time to receive a TCR-mediated signal to trigger CD4 lineage development. This possibility suggests a more indirect function for E-proteins. The loss of E-proteins may not normally occur immediately upon receiving a positive selection signal. Properly timed down-regulation of E-protein activity may allow for a developmental window during

which cells can interpret lineage determining signals before the DP to SP transition is completed. This possibility is supported by HSA staining of  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+$  thymocytes (Figure 26). SP cells in  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+$  mice reveal a DP-like high level of surface HSA, suggesting that differentiation from DP to SP or SP maturation may occur at a faster rate. Regardless, both scenarios suggest that lineage instruction may be initiated prior to complete down-regulation of E-protein activity.

If E-proteins are required for initiating CD4 lineage instruction, enforced expression of Th-POK should be able to rescue the CD4 population in  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+$  mice. We plan to test this hypothesis by creating a transgenic mouse containing Th-POK cDNA preceded by a floxed stop cassette. Crossing this transgene onto our  $Tcf12^{f/f}Tcfe2a^{f/f}CD4Cre^+$  background will allow activation of the Th-POK cDNA along with HEB and E2A deletion in DP thymocytes. A rescue of CD4 cells would indicate that E-proteins function upstream of Th-POK initiation. If we do not see a rescue of CD4 cells, E-proteins may function downstream or in parallel with Th-POK. However, if we do not see a rescue in the CD4 population, we must first ensure that Gata3 and CD4 are properly expressed. These analyses, along with more detailed investigation of the kinetics of DP to SP development, will hopefully allow us to begin placing E-proteins into or reshaping the current models for CD4 and CD8 lineage development.

Another remaining issue is to determine the connection between TCR signaling and down-regulation of E-protein function. As mentioned previously, one suggested mechanism is the inhibition of E-protein activity by induction of Id3 (Bain et al., 2001). Id3 transcription is induced downstream of ERK MAPK signaling through activation of the transcription factors SAP-1 and Egr1 (Costello et al., 2004). Id3 can then inhibit E-protein DNA binding activity by competitive dimerization. Even though Id3 deficient mice demonstrate a reduction in positively selected cells, positive selection is not completely blocked (Rivera et al., 2000). Therefore, there must be additional mechanisms driving the positive selection signal in addition to Id3 induction. Is E-protein down-regulation required for positive selection? If so, is Id2 able to compensate for the absence of Id3 to drive positive selection? To begin addressing these questions we plan to delete both Id3 and Id2 at the DP stage. As with the deletion of both HEB and E2A, we anticipate deletion of both Id3 and Id2 to reveal novel functions previously masked in single knockout animals.

In addition to Id induction, there may be other signaling pathways contributing to TCR-mediated E-protein down-regulation. Calcium signaling has been suggested to result in reduced E-protein DNA binding activity through the activation of the calcium binding protein calmodulin. Calcium loaded calmodulin has been shown to bind and block DNA binding activity of E-proteins (Saarikettu et al., 2004). It is possible that calcium/calmodulin activity provides the first wave of E-protein inhibition, followed by

the induction of Id3 to further block E-protein function. While these mechanisms provide examples of inhibition of E-protein activity, we do not yet understand how E-proteins are also down-regulated at the transcriptional level.

One of the other overall goals of the studies presented in this dissertation is to identify new E-protein target genes and co-factors. The discovery of novel targets and interacting proteins is anticipated to help address major questions remaining in the field. A few of the following issues have also been reviewed in one of my previous publications (Jones and Zhuang, 2008). For example, what are the underlying mechanisms guiding E-protein directed transcriptional regulation in a lineage and stage specific manner? E-protein lineage and stage specific effects can be regulated by interactions with different binding factors exhibiting more restricted patterns of expression. For example, in the B cell lineage, E2A cooperates with EBF1 and Pax5 to regulate a set of its B cell specific target genes (discussed in Chapter 1), and interaction with IRF4 has been shown to promote E2A recruitment at the Ig $\kappa$  3' enhancer in pre-B cells (Lazorchak et al., 2006a). So far only a few other co-activators interacting with E-proteins in lymphocytes have been identified. For example, the histone acetyltransferases (HATs) p300, CBP, and PCAF have been identified to associate with E-proteins (Bradney et al., 2003; Eckner et al., 1996; Massari et al., 1999; Qiu et al., 1998). One study demonstrates that these interactions can occur in B cells, and HATs are able to enhance E2A transcriptional activity (Bradney et al., 2003). However, the E2A target

genes dependent on E2A-HAT associations have not yet been defined. In addition to associations with activating complexes, E-proteins have also been shown to interact with transcriptional repressors. For example, the co-repressor ETO can interact with E-proteins and in doing so, blocks the recruitment of HATs (Zhang et al., 2004). ETO is also able to bind histone deacetylases (HDACs) (Hug and Lazar, 2004; Zhang et al., 2004). Thus, E-protein recruitment of HATs versus HDACs could contribute to the variations in E-protein function in different lineages and stages of development. Lineage and stage specific functions of E-proteins are likely to be determined by several levels of regulation. In addition to altering associated binding factors, differences in E-protein dosage and accessibility to E-box sites can further determine the downstream pathways activated by E-proteins.

Another remaining issue of interest is to determine what roles E-proteins play in addition to directly activating and repressing gene expression. Along with the studies showing E-protein associations with HATs and HDACs, multiple other studies have suggested that E-proteins can regulate chromatin modification. For example, E2A is suspected to generate localized accessibility around specific gene segments within the Ig receptor loci (Goebel et al., 2001), and E2A, EBF1, and Pax5 have been shown to regulate CpG demethylation and nucleosome remodeling at the *mb-1* promoter (Maier et al., 2004). Additionally, E2A has been shown to play a role in Ig $\kappa$  enhancer acetylation (Lazorchak et al., 2006a).

Even though E-proteins are DNA binding factors, it is important to keep in mind that they may also serve a function in addition to acting as transcription factors. The interaction of E-proteins with Id proteins may provide an example of a function independent of interactions with DNA and chromatin remodeling complexes. Id proteins are considered regulators of E-protein DNA binding activity. However, the interaction between Id and E-proteins may also provide regulation of Id activity. While their primary function is clearly to regulate E-protein function, Id proteins are suggested to also interact with and interfere with the function of other transcription factors, for example Pax5 and members of the ETS-domain transcription factor family (Roberts et al., 2001; Yates et al., 1999). E-proteins may therefore be sequestering Id proteins to regulate interactions of Id proteins with alternative binding factors.

The ability of E-proteins and Id proteins to bind multiple factors further highlights the importance of timing and dosage of gene expression. Differentiation through the stages of lymphocyte development, or even more general cell processes, are likely to be dependent on the fine balance of these factors. The genetic models presented in this dissertation provide new tools for manipulating the timing of E-protein function and the dosage of E-protein expression. These methods used here may also be applied to manipulating the expression of Id and other relevant transcription factors. Future studies involving this new generation of animal models should aid identification of

novel target genes, binding factors, and the upstream pathways regulating E-protein activity.

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### Publications

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### Fellowships

2003 James B. Duke Fellowship, Duke University Graduate School