

THE ROLE OF HEB AND E2A IN THE REGULATION OF T LYMPHOCYTE  
DEVELOPMENT AND PROLIFERATION

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Dissertation submitted in partial fulfillment  
of the requirements for the degree of Doctor of Philosophy  
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2007

ABSTRACT

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## ABSTRACT

Thymocyte development is a complex process that requires precise regulation of differentiation and proliferation. Basic helix-loop-helix (bHLH) transcription factors have been shown to be crucial for proper T cell development. HEB and E2A are structurally and functionally related E proteins of the bHLH family. These proteins directly regulate the expression of a number of genes essential for lymphocyte development in a lineage- and stage-specific manner. Abrogation or compromise of their function results in the manifestation of B and T cell developmental defects.

Genetic and biochemical studies have provided evidence of a significant degree of functional redundancy among E proteins. The existence of compensational abilities among different E proteins has hampered the investigation and elucidation of E protein function. As such, single gene knockouts demonstrate only limited defects in lymphocyte development. Double E2A-HEB knockouts that could eliminate E protein redundancy are embryonic lethal. In addition, conventional gene knockouts are not well-suited for discerning between intrinsic and extrinsic defects caused by E protein disruption.

To eliminate functional compensation and to test the T cell intrinsic roles of E proteins during thymocyte development, we developed a conditional HEB-E2A double knockout. Specifically, we employed a loxP/*Lck*-Cre recombinase system to drive E protein deletion during early thymocyte development. Using this approach, we were able

to reveal overlapping roles for HEB and E2A in thymocyte development that had been obscured in previous single gene knockout studies.

We find that simultaneous deletion of HEB and E2A results in a severe block in thymocyte development at the DN to DP stage transition. This developmental block is accompanied by a dramatic decrease in total thymic cellularity, an increase in apoptosis, and a reduction of pT $\alpha$  expression. These developmentally arrested thymocytes exhibit increased proliferation *in vivo* and dramatic expansion *ex vivo* in response to IL-7 signaling. Our findings suggest that E2A and HEB are not only critical for the regulation of T cell differentiation but are also necessary to retain developing thymocytes in cell cycle arrest prior to pre-TCR expression. Together, these results imply that E proteins are required to coordinate thymocyte differentiation and proliferation.

## **DEDICATION**

This dissertation is dedicated to my parents, Donna and the late Robert Wojciechowski

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## LIST OF ABBREVIATIONS

- 7AAD: 7-aminoactinomycin D
- APC: allophycocyanin
- bHLH: basic-helix-loop-helix
- CDK: cyclin-dependent kinase
- CDKI: cyclin-dependent kinase inhibitor
- ChIP: chromatin immunoprecipitation
- CLP: common lymphoid progenitor
- D: diversity gene segment
- DC: dendritic cell
- DKO: E2A-HEB double conditional knockout
- DN: double-negative
- DP: double-positive
- EMSA: electrophoretic mobility shift assay
- ES: embryonic stem cells
- E2A<sup>loxP</sup>: targeted E2A allele flanked by two loxP sites
- FACS: fluorescence-activated cell sorter
- FBS: fetal bovine serum
- FITC: fluorescein isothiocyanate
- FOXO1: forkhead transcription factor 1

GSK3 $\beta$ : glycogen synthase kinase 3 $\beta$

HEB: HeLa E-box binding transcription factor

HEB<sup>bm</sup>: dominant negative HEB allele containing a basic region mutation

Id: inhibitor of DNA binding/ inhibitor of differentiation

IL: interleukin

J: joining gene segment

Jak: Janus family kinase

LAT: linker for the activation of T cells

Lck-Cre: Cre recombinase driven by the *lck* proximal promoter

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

pT $\alpha$ : pre-T $\alpha$

PE: phycoerythrin

PI3K: phosphatidylinositol 3-kinase

Pre-TCR: pre-T cell receptor

Pten: phosphatase and tensin homolog

PTK: protein tyrosine kinase

RAG: recombinase-activating gene

SKO: HEB single conditional knockout

SP: single-positive

STAT: signal transducer and activator of transcription

TC: tricolor

TCR: T cell receptor

V: variable gene segment

WT: wild type



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**CHAPTER 1**  
**INTRODUCTION**

## **1.1 T lymphocyte Development**

### **1.1.1 $\alpha\beta$ T cell differentiation**

T cells represent an essential component of the adaptive immune system. Two main classifications of T cells exist in mammals:  $\alpha\beta$  T cells and  $\gamma\delta$  T cells. This designation is based on the respective  $\alpha\beta$  or of  $\gamma\delta$  heterodimeric T cell receptor (TCR) displayed on the cell surface. Important functional differences are conveyed onto the T cell depending on which of the two T cell receptors is expressed. T cells of the  $\alpha\beta$  lineage represent the vast majority of peripheral T cells and are typically viewed as conventional T cells. In contrast,  $\gamma\delta$  T cells are much fewer in number and serve specialized functions in the host in particular locations such as the intestinal and pulmonary epithelium and the skin (Lauritsen et al., 2006).

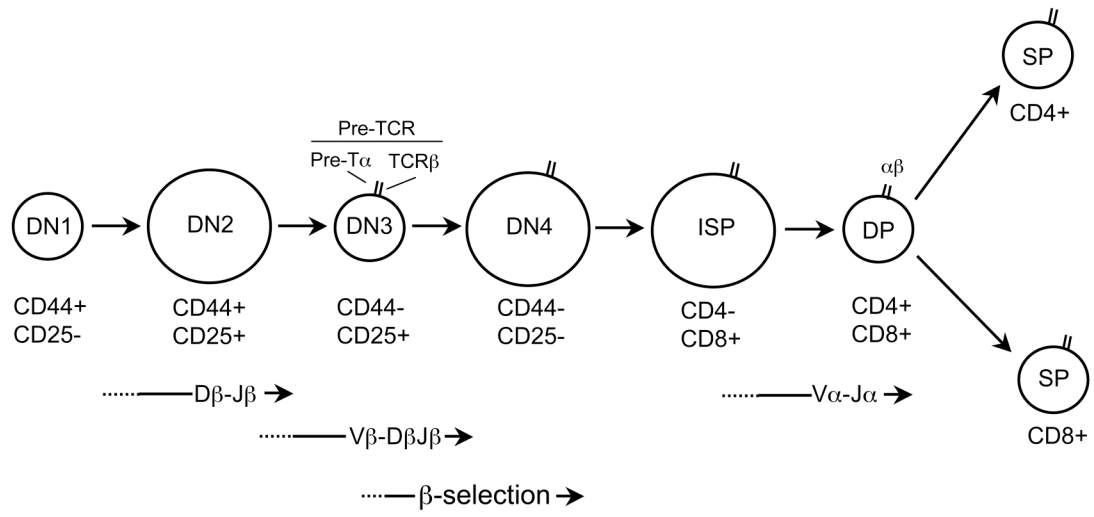
T cells originate from long-lived, self-renewing hematopoietic stem cells (HSCs). This small population of lineage-negative pluripotent cells resides in the fetal liver during gestation and then the bone marrow after birth. HSCs give rise to progressively more restricted multipotent lineage progenitors of the lymphoid and myeloid cell lineages. These cells are termed the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), respectively. These progenitors are then in turn able to generate all mature blood cell types including B and T cells, NK cells, granulocytes, macrophages, megakaryocytes, and erythrocytes (reviewed by Kondo et al., 2003).

T lymphocyte development ensues in the thymus upon the arrival of bone marrow-derived progenitors. Once these precursors enter the thymus, T cell differentiation follows a highly ordered and tightly regulated progression of maturational

steps. This sequential developmental progression is accompanied by changes in cell surface marker expression, TCR recombination status, and functional capabilities. In brief, cells begin at the CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) stage of development and progress through the CD8<sup>+</sup> immature single-positive (ISP) then the CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage to eventually reach the mature CD4<sup>+</sup> or CD8<sup>+</sup> single-positive (SP) stage of development (Figure 1.1). Critical to successful T cell maturation is the acquisition and expression of a functional TCR at discrete developmental steps. T cell receptor  $\beta$  (TCR $\beta$ ) and TCR $\alpha$  gene segments recombine at the DN and DP stage of development, respectively, to form a mature clonotypic  $\alpha\beta$ TCR. The TCR $\beta$  chain is formed by the joining of variable (V), diversity (D), and joining (J) gene segments while the TCR $\alpha$  chain consists of only V and J segments. In mice, there are  $\sim 25$  V $_{\beta}$ , 2 D $_{\beta}$ , and 12 J $_{\beta}$  gene segments that recombine to generate the TCR $\beta$  chain. The TCR $\alpha$  chain can be produced from  $\sim 100$  V $_{\alpha}$  and 60 J $_{\alpha}$  gene segments. Proper rearrangement of both the TCR $\beta$  and TCR $\alpha$  chains constitute important developmental checkpoints during maturation of  $\alpha\beta$  lineage T cells.

DN cells can be further divided into four subsets based on their expression of CD44 (hyaluronic acid receptor) and CD25 (IL-2R $\alpha$ ) such that they begin at the DN1 (CD44<sup>+</sup>CD25<sup>-</sup>), and progress through the DN2 (CD44<sup>+</sup>CD25<sup>+</sup>), DN3 (CD44<sup>-</sup>CD25<sup>+</sup>), and DN4 (CD44<sup>-</sup>CD25<sup>-</sup>) stages (Godfrey et al., 1993). DN1 cells are not committed to the T cell lineage and have not yet begun TCR recombination. These cells are multipotent and capable of differentiation into T, B, NK, or dendritic cells (DCs) (Ardavin et al., 1993; Godfrey et al., 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995). Following

**Figure 1.1 Overview of  $\alpha\beta$  T lymphocyte development.** Cells progress through the DN1-4, DP, and SP thymocyte subsets to generate mature  $\alpha\beta$  T cells. The cell surface phenotypic markers CD44, CD25, CD4, and CD8 are used to discern among the different stages of differentiation. The recombination status of the TCR $\alpha$  and  $\beta$  chains during development is shown. At the DN3 stage, PT $\alpha$  pairs with the TCR $\beta$  chain to produce the pre-TCR, which initiates  $\beta$ -selection. At the DP stage the PT $\alpha$  chain is replaced with the TCR $\alpha$  chain to generate a mature  $\alpha\beta$  TCR. Large and small circles represent proliferating and quiescent non-cycling cells, respectively.



progression to the DN2 stage of development, thymocytes begin  $\alpha\beta$  T cell lineage restriction and lose their B cell differentiation potential (Godfrey et al., 1994; Moore and Zlotnik, 1995; Wu et al., 1996). At this stage, recombinase-activating gene 1 (RAG1) and RAG2 are induced (Godfrey and Zlotnik, 1993) and rearrangement of  $D_\beta$  to  $J_\beta$  gene segments within the TCR $\beta$  locus commences.

Once cells reach the DN3 stage of development, they are considered fully committed to the T cell lineage and  $V_\beta$  segments recombine with  $D_\beta J_\beta$  gene products to form the mature TCR $\beta$  chain (Fehling et al., 1995; Godfrey et al., 1994; Godfrey et al., 1993). Expression of a functional TCR $\beta$  chain constitutes a critical developmental checkpoint during the DN stage of thymocyte development. Only in-frame TCR $\beta$  rearrangements are able to produce a functional TCR $\beta$  chain, which is expressed on the cell surface paired with an invariant surrogate alpha chain (pT $\alpha$ ) to form the pre-T cell receptor (pre-TCR). Signaling through this receptor complex and its associated CD3 signal transducing components triggers beta selection and allelic exclusion, which arrests further TCR $\beta$  rearrangement, induces rapid cell proliferation, and allows for the progression to the DN4 and DP stages of development (von Boehmer et al., 1999). Accordingly, mice deficient in TCR $\beta$  recombination, pT $\alpha$ , or pre-TCR signaling manifest a block at the DN3 stage of development (Fehling et al., 1995; Mombaerts et al., 1992a; Mombaerts et al., 1992b).

At the double positive CD4<sup>+</sup>CD8<sup>+</sup> stage of development, recombination of  $V_\alpha$  and  $J_\alpha$  gene segments occurs to generate the TCR $\alpha$  chain. Following successful TCR $\alpha$  rearrangement, the TCR $\alpha$  chain is expressed on the cell surface with the TCR $\beta$  chain to



form the mature  $\alpha\beta$ TCR. Once a mature TCR is expressed, the critical biological processes of positive and negative selection shape the T cell repertoire such that only a small percentage of cells containing a  $\alpha\beta$ TCR capable of recognizing self-MHC (major histocompatibility complex)/ antigenic peptide complexes with the appropriate avidity and specificity are allowed to continue maturation. Cells that do not recognize self-MHC complexes or those with too high an affinity are not positively selected and are deleted by negative selection. The stringency of this process is such that approximately 97% of cells in the thymus die by apoptosis (Egerton et al., 1990). Successful completion of these events results in functionally mature  $CD4^+$  or  $CD8^+$  T cells that are well-suited for response to foreign antigen presentation in the periphery.

### **1.1.2 Thymocyte proliferation during development**

Proliferation and differentiation are coordinately regulated during thymocyte development. During the DN stages of development, cells must balance cell cycle progression with ongoing rearrangement of their TCR $\beta$  chain. It is generally thought that proliferation and differentiation are mutually exclusive events (Alema and Tato, 1987). Therefore, the regulation of proliferation vs. differentiation is a critical event during T cell development because inappropriate proliferation during recombination (or vice versa) may reduce the efficiency of successful T cell development. For instance, the cell may interpret RAG-induced double-stranded DNA breaks during TCR $\beta$  recombination as DNA damage and undergo apoptosis if cell cycle progression is initiated simultaneously (Kim et al., 1999).

Two distinct rounds of proliferation occur during DN thymocyte development (Figure 1.1). As cells reach the DN2 stage of differentiation, the first burst of expansion occurs. This transient proliferation is followed by a slow or non-proliferating phase as cells enter the DN3 stage of development (Penit et al., 1995; Tourigny et al., 1997). These early DN3 thymocytes are retained within the G<sub>1</sub> phase of cell cycle prior to pre-TCR expression (Hoffman et al., 1996). These small non-blasting cells retain RAG2 expression and undergo V<sub>β</sub>-D<sub>β</sub>J<sub>β</sub> rearrangements of the TCRβ chain. Upon the successful expression of the pre-TCR and completion of beta selection, RAG-2 expression is downregulated, cell cycle arrest is released, and proliferation ensues (Hoffman et al., 1996; Laurent et al., 2004). From progenitor migration to the thymus to the DN3 stage of development, an estimated 9-10 cell divisions occur. This corresponds to approximately a 1000-fold expansion of thymocyte numbers. The presumed function of this proliferation is to generate a large population of DN3 cells to maximize the chances of successful in-frame and diverse TCRβ rearrangements (Penit et al., 1995; Shortman and Wu, 1996). Following successful β-selection, thymocytes undergo another 6-7 rounds of proliferation (Penit et al., 1995). This cell expansion analogously yields a large pool of DP cells for TCRα recombination.

Importantly, the mechanisms responsible for inducing proliferation at these distinct developmental stages are different. Proliferation at the DN2 stage is dependent on IL-7 signaling (Akashi et al., 1997; Kim et al., 1998; Peschon et al., 1994; von Freuden-Jeffrey et al., 1997). In contrast, proliferation of late DN3 cells is IL-7 independent. In these cells, pre-TCR signaling alone is necessary and sufficient for

proliferation, survival, and continued maturation (Fehling et al., 1995; Mombaerts et al., 1992a; Mombaerts et al., 1992b; Shinkai et al., 1992).

## **1.2 Regulation of Cell Cycle**

Cell cycle is divided into two main stages: mitosis (M) and interphase. These correspond to the stages in which cells undergo cellular division, and DNA replication, respectively. Mitosis is further broken up into prophase, metaphase, anaphase and telophase. The purpose of mitosis is to ensure equal partitioning of genetic material to daughter cells during cell division. Interphase consists of a G<sub>1</sub> phase (the first gap), the S (DNA synthesis) phase, and a G<sub>2</sub> phase (second gap). During the G<sub>1</sub> and G<sub>2</sub> phases the cell prepares for DNA synthesis (S phase) and mitosis, respectively. Quiescent cells that have exited cell cycle after M phase but prior to entry into interphase are in the G<sub>0</sub> or resting phase.

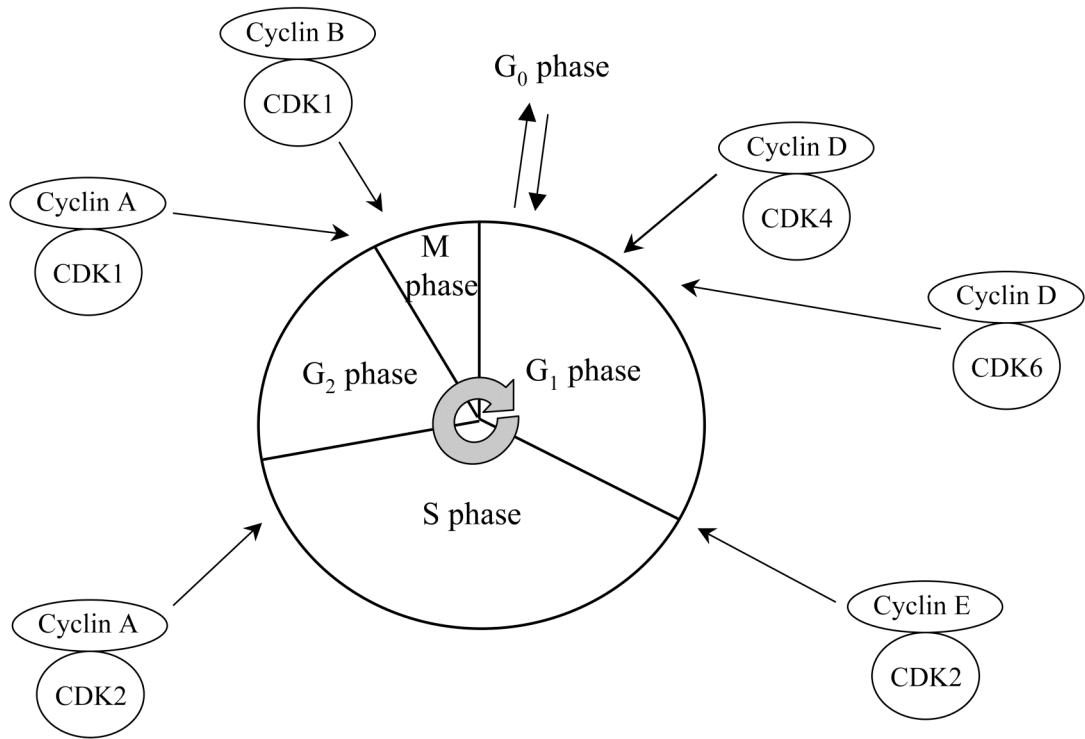
Progression through the cell cycle is a tightly regulated biological process. The decision to advance or arrest cell cycle is controlled by the balance of factors that promote and those that inhibit cycle cell. These correspond to cyclins and cyclin-dependent kinase inhibitors (CDKIs), respectively (Kozar et al., 2004; Sherr and Roberts, 1999). Cyclins bind and subsequently activate cyclin-dependent kinases (CDKs). CDKs are a group of serine/threonine protein kinases that once activated, phosphorylate key downstream targets allowing for cell cycle progression. Four CDKs have been identified that are active in cell cycle. These are CDK2, CDK4, and CDK6 which function in G<sub>1</sub> phase, CDK2 which is active in S phase, and in CDK1 in G<sub>2</sub> phase. While expression of

CDKs is stable throughout cell cycle, expression of cyclins (as their name implies) fluctuates.

A number of different cyclins are able to form complexes with CDKs and perform phase-specific regulatory duties during cell cycle (reviewed by (Vermeulen et al., 2003) Vermeulen et al., 2003) (Figure 1.2 and Table 1.1). Cyclin D-CDK4/6 complexes regulate G<sub>1</sub> phase entry (Sherr, 1994). As cell cycle progresses, cyclin E-CDK2 complexes are involved in the G<sub>1</sub> to S phase transition (Ohtsubo et al., 1995). Once cells enter S phase, cyclin A-CDK2 complexes are required for completion of DNA replication (Girard et al., 1991; Walker and Maller, 1991). During G<sub>2</sub> phase cyclin A-CDK1 complexes regulate entry into mitosis and cyclin B-CDK1 complexes regulate M phase progression (King et al., 1994).

CDKIs act to inhibit the activity of CDKs and thus arrest cell cycle progression. Two families of CDKIs exist: the INK4 family and the Cip/Kip family. The former includes the members p15 (INK4b), p16 (INK4a), p18 (INK4C), and p19 (INK4D). These proteins function in G<sub>1</sub> phase by binding CDK4 and CDK6, thus prohibiting their ability to form complexes with D cyclins. The Cip/Kip family includes p21 (Waf1, Cip1), p27 (Cip2), and p57 (Kip2). Members of this family also inhibit G<sub>1</sub> phase progression but do so by direct binding to cyclin D-CDK complexes.

**Figure 1.2 Overview of cell cycle and phase-specific associated cyclin/CDK complexes.** Cell cycle is divided into G<sub>1</sub> phase, S phase, G<sub>2</sub> phase, and M phase. The phase at which cyclin-CDK complexes function to regulate cell cycle is indicated. Adapted from Vermeulen et al., 2003.



**Table 1.1 Specific cyclin-CDK complexes are active in a phase-specific manner during cell cycle.** Adapted from Vermeulen et al., 2003.

Phase of Cell Cycle	CDK	Cyclin
G <sub>1</sub> phase	CDK4	Cyclin D1, D2, D3
G <sub>1</sub> phase	CDK6	Cyclin D1, D2, D3
G <sub>1</sub> /S phase transition	CDK2	Cyclin E
S phase	CDK2	Cyclin A
G <sub>2</sub> /M phase transition	CDK1	Cyclin A
M phase	CDK1	Cyclin B



## **1.3 Role of E proteins in T cell Development**

### **1.3.1 Basic helix-loop-helix (bHLH) transcription factors**

Basic helix-loop-helix (bHLH) proteins are a broad class of transcription factors originally characterized for their involvement in cellular differentiation and lineage determination (Jan and Jan, 1993; Olson and Klein, 1994). As their name implies, members of the bHLH family share two important conserved structural components: a basic region that allows for DNA-binding and an adjacent HLH domain comprised of two amphipathic  $\alpha$ -helices separated by a loop structure that allows for dimerization with other bHLH proteins (Murre et al., 1989b; Voronova and Baltimore, 1990).

Three classes of bHLH transcription factors exist in mammals (Benezra et al., 1990; Murre et al., 1989b). The first class is composed of the E proteins E2A, E2-2, and HEB (HeLa E box-binding factor). The E2A gene encodes the products E12 and E47, which are generated by alternate splicing of adjacent bHLH exons (Kamps et al., 1990; Murre et al., 1989a). From this point forward, we will use E2A to refer to both E12 and E47 alleles, unless otherwise specified. Class I bHLH transcription factors are broadly expressed in a variety of cell lineages and form functional homo- or heterodimers with other bHLH class members (Henthorn et al., 1990; Hu et al., 1992). E protein structure is illustrated in Figure 1.3A. Class II bHLH proteins include tissue-specific factors that only function in a lineage-autonomous manner. Examples include MyoD in muscle cells (Weintraub et al., 1991; Weintraub et al., 1989) and Add1 in adipocytes (Tontonoz et al., 1993). These factors typically have a higher affinity for E protein heterodimerization

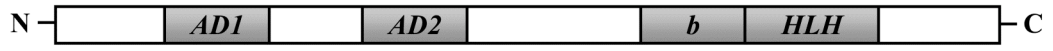
than self-dimerization (Lassar et al., 1991; Murre et al., 1989b). The third class includes the broadly expressed Id (Inhibitor of Differentiation/Inhibitor of DNA-binding) proteins (Benezra et al., 1990). In contrast to other bHLH class members, these proteins lack a basic DNA-binding domain. This prevents any Id-containing protein complex from binding DNA and thus functions as a dominant-negative by competitive dimerization. Id proteins preferentially bind class I (E) proteins over class II (Benezra et al., 1990; Sun et al., 1991). Although four Id genes (Id1-4) exist in mammals, only Id2 and Id3 are expressed in lymphocytes (Cooper et al., 1997; Massari and Murre, 2000).

### **1.3.2 E proteins in thymocyte development**

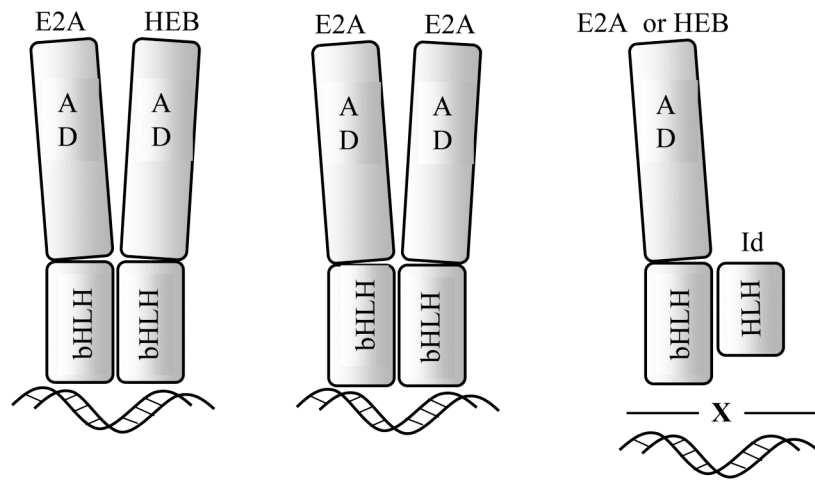
E proteins were originally identified as Ig $\kappa$  light chain enhancer binding proteins (Murre et al., 1989a). Since this initial observation, E proteins have been recognized as being essential for lymphocyte development. As homo- or heterodimers E proteins bind to DNA at sites termed E boxes represented by the sequence CANNTG, and stimulate target gene transcription (Figure 1.3B). During development, E proteins regulate several important lineage-specific target genes in a stage-specific manner. In B cells these targets include the Ig $\kappa$  3' and intronic enhancers, IgH 3' and intronic enhancers, Early B cell factor (EBF) 5' locus, mb-1 (Ig $\alpha$ ) promoter, B29 (Ig $\beta$ ) 5' locus,  $\lambda$ 5 promoter, VpreB promoter, TdT 5' locus, and the RAG-2 enhancer (Choi et al., 1996; Greenbaum et al., 2004; Greenbaum and Zhuang, 2002; Hsu et al., 2003; Murre et al., 1989a; Schlissel et al., 1991; Sigvardsson, 2000; Sigvardsson et al., 1997). In T cells, E proteins have been

**Figure 1.3 Schematic diagram E protein structure and function.** (A) E proteins contain two N-terminal transactivation domains (AD1 and AD2). These domains are thought to contribute to protein-protein interactions and transcriptional regulation. They are followed by a basic (b) DNA-binding domain and an adjacent helix-loop-helix (HLH) motif responsible for protein dimerization. (B) E proteins dimerize with different partners depending on the cell type and bind DNA at E boxes. In B cells E2A homodimers form the predominant functional dimer species, while in T cells HEB:E2A heterodimers are thought to be the main E protein pair. Id proteins lacking the basic DNA-binding region can also dimerize with E proteins, however these complexes are unable to bind DNA. Part B adapted from Lazorchak et al., 2005.

**A**



**B**



shown to regulate the TCR $\beta$  enhancer, the pT $\alpha$  promoter and enhancer, and various CD4 regulatory elements (Greenbaum and Zhuang, 2002; Sawada and Littman, 1993; Takeuchi et al., 2001). Also, the promoter for the IL-7 gene contains a putative E12 binding site (Lupton et al., 1990).

In contrast to E47 homodimers which are the main contributor of E protein function for B cell development in the bone marrow, E2A:HEB heterodimers form the predominant E box binding species in the thymus during T cell development (Sawada and Littman, 1993; Shen and Kadesch, 1995). Interestingly, the constituents of the dimer pair may influence lineage-specific gene regulation, as ectopic overexpression of E12 or E47 in non-B cell lines results in the induction of a number of B lineage-restricted genes and Ig locus rearrangement (Kee and Murre, 1998; Schlissel et al., 1991). Also, although a substantial degree of redundancy is present among different E proteins, specific bHLH pairs have been shown to be capable of discerning between closely related E box sites and thus adds another dimension of regulation to target gene expression (Hu et al., 1992; Lassar et al., 1991).

A variety of experimental approaches have been taken in an effort to elucidate the role of E proteins in thymocyte development (Table 1.2). Manipulation of E protein function by conventional gene knockouts (Bain et al., 1997; Barndt et al., 1999), conditional knockouts (Pan et al., 2002), dominant-negative strategies (Barndt et al., 2000), and genetic locus exchange approaches (in which one E protein gene is inserted into another's genomic locus) (Zhuang et al., 1998), have contributed to our

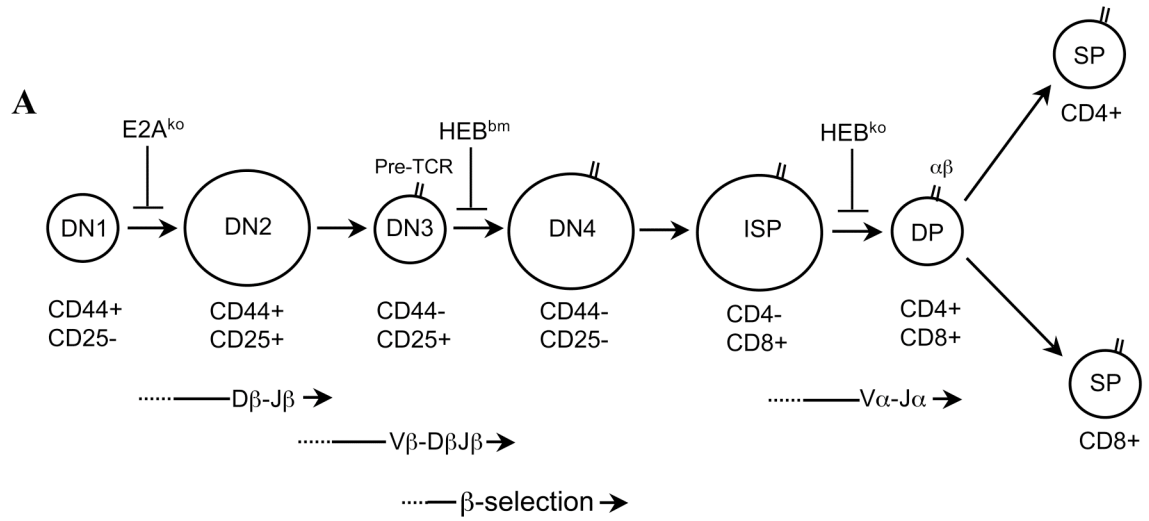
**Table 1.2 Summary of E protein disruption models as they pertain to T cell development.** Reviewed are E2A<sup>ko</sup>, HEB<sup>ko</sup>, E2-2<sup>ko</sup>, HEB<sup>bm</sup>, and conditional E2A<sup>ko</sup>. E47<sup>bm</sup> and E2A<sup>HEB</sup> mice are not included because no T cell phenotype was described in the initial publication (Zhuang et al., 1998).

	E2A <sup>ko</sup>	HEB <sup>ko</sup>	E2-2 <sup>ko</sup>	HEB <sup>bm</sup>	conditional E2A <sup>ko</sup>
T cell development	Partial DN1 block	ISP block	Normal	DN3 block	Normal
Thymic cellularity	5-10-fold decrease	5-10-fold decrease	Normal	15-100-fold decrease	Normal
Thymoma development	Yes	Yes	NR	Yes	No
Viability	Neonatal death	Neonatal death	Neonatal death	Neonatal death	Normal
Fertility	Male: reduced fertility Female: sterile	Sterile	NR	NR	Normal
Reference	Zhuang et al., 1994 Zhuang et al., 1998	Barndt et al., 1999	Zhuang et al., 1996 Bergqvist et al., 2000	Barndt et al., 2000	Pan et al., 2002

NR: Not reported

**Figure 1.4 Overview of E proteins and  $\alpha\beta$  T lymphocyte development.** (A) Cells progress through the DN1-4, DP, and SP thymocyte subsets to generate mature T cells. The cell surface phenotypic markers CD44, CD25, CD4, and CD8 are used to discern among the different stages of differentiation. The recombination status of the TCR $\alpha$  and  $\beta$  chains during development is shown. At the DN3 stage, PT $\alpha$  pairs with the TCR $\beta$  chain to produce the pre-TCR which initiates  $\beta$ -selection. At the DP stage the PT $\alpha$  chain is replaced with the TCR $\alpha$  chain to generate a mature  $\alpha\beta$  TCR. Large and small circles represent proliferating and quiescent non-cycling cells, respectively. The different stages at which disruption of E proteins blocks thymocyte development are indicated. (B) Expression of HEB and E2A during DN1-4 subsets is indicated. Expression as shown is not comparable between HEB and E2A but rather is representative of stage-specific changes for that specific E protein.





**B**

	DN1	DN2	DN3	DN4
E2A	+	++++	+++++	+++++
HEB	+	++	+++++	+++++

understanding of E protein function and result in a broad range of defects in lymphocyte development.

Mice lacking a functional E2A gene exhibit a partial block in thymocyte development at the DN1 stage before the onset of TCR $\beta$  rearrangement or lineage commitment and have 5-10-fold reduced thymic cellularity (Figure 1.4A and Table 1.2). E2A also plays a role at later stages of development, as E2A<sup>-/-</sup> mice display an accelerated transition from the DP to SP stage of T cell development (Bain et al., 1999a; Kee et al., 2002). In addition, E2A-deficient mice display a high frequency of thymic derived T cell leukemia (Bain et al., 1997; Engel and Murre, 2002; Yan et al., 1997). HEB knockout mice are similar to E2A deficient mice in terms of reduced thymic cellularity, but display a developmental arrest at the CD8 immature single-positive (ISP) stage, a transitional stage between the DN and DP stages of thymocyte development (Barndt et al., 1999) (Figure 1.4A and Table 1.2). E2A/HEB compound heterozygous mice demonstrate an accumulation of ISP cells, a defect similar to that seen in the HEB<sup>ko</sup> (Zhuang et al., 1996). This observation supports the notion that the combined dosage of E proteins is required for proper T cell development.

As previously mentioned, Id proteins antagonize E protein function by the formation of non-functional dimers. While the disruption of individual Id genes generally causes little or no T cell developmental defects (Pan et al., 1999; Yan et al., 1997; Yokota et al., 1999), ectopic expression of Id proteins in thymocytes results in severe developmental consequences. T cell-specific transgenic Id1 mice demonstrate a strong block at the DN1 stage of development, a severe induction of apoptosis, and a

100-fold decrease in T cell numbers. In addition these mice develop T cell lymphoma (Kim et al., 1999). Overexpression of Id3 in thymocytes results in similar developmental consequences, including a block in T cell differentiation and enhanced proliferation of thymocytes (Heemskerk et al., 1997; Morrow et al., 1999).

### **1.3.3 Expression and activity of E proteins during T cell development**

The regulation of E protein expression in a stage-specific manner is important during lymphocyte development to ensure appropriate target gene activation. E protein expression is not constant during T cell development and varies considerably according to the stage of differentiation (Figure 1.4B). Importantly, the timing and levels of E2A and HEB expression in T cells are distinct. Such differences may contribute to the distinct phenotypic defects observed in HEB and E2A knockouts. E2A protein is expressed at high levels in hematopoietic stem cells (HSCs) (Zhuang et al., 2004). As cells enter the DN1 stage, they express low but detectable levels of E2A. Once cells enter the DN2 stage E2A expression is dramatically upregulated. This increase in E2A expression correlates with the onset of TCR $\beta$  recombination and T lineage commitment. Levels of E2A expression remain elevated through the DN3 and DN4 subsets. Expression is then downregulated to moderate levels in DP cells and is further reduced upon advancement to the SP stage (Engel et al., 2001; Pan et al., 2002).

Expression of HEB is turned on later than E2A in the DN stages of thymocyte development. In contrast to E2A, stage-specific HEB protein expression has not been examined, thus we are currently limited to mRNA analyses. HEB expression is low in

DN1 thymocytes and increases 2-fold to moderate levels in DN2 cells. Upon the entrance into the DN3 compartment, HEB expression is dramatically upregulated. During this stage the critical developmental processes of  $V_{\beta}$ - $D_{\beta}$  $J_{\beta}$  rearrangement and pre-TCR expression occur. This high level of HEB expression is maintained in DN4 cells (Taghon et al., 2006) (Figure 1.4B).

The regulation of E2A and HEB expression likely occurs at both the transcriptional and posttranscriptional levels and may be different between the two E proteins. Although protein levels of E2A have been shown to fluctuate during the different stages of T cell development, E2A mRNA transcript levels remain relatively constant (Engel et al., 2001). Also, while E2A protein levels in pre-B cells are higher than in pro-B cells, mRNA levels are similar (Riley et al., 2002). Posttranscriptional modifications that have been shown to potentially affect E protein function and levels include acetylation, phosphorylation, and ubiquitination. These modifications can alter E protein stability, DNA binding, and transcriptional activity (Bradney et al., 2003; Huggins et al., 1999) (Y. Zhuang unpublished observations). For instance, Notch signaling can target E47 for degradation by ubiquitination (Huang et al., 2004). Finally, dimerization status dramatically influences E protein activity. This is accomplished by the formation of Id-E protein dimers, which are unable to bind DNA and are transcriptionally inactive. The combination of transcriptional and posttranscriptional regulation endows the cell with multiple ways to manipulate E protein activity to ensure proper functioning.

### 1.3.4 Functional redundancy of E proteins

Genetic and biochemical studies have provided evidence of a significant degree of functional redundancy among E proteins (Barndt et al., 1999; Barndt et al., 2000; Zhuang et al., 1998). For example, targeting of HEB cDNA into the E2A genomic locus (E2A<sup>HEB</sup>) allows relatively normal B cell development in the absence of E2A (Zhuang et al., 1998). Compensation is manifested at the protein expression level as well. E2A protein levels were shown to be increased 2-fold in HEB<sup>ko</sup> mice (Barndt et al., 1999). Finally, functional examination of dimer formation by EMSA using a canonical T cell E box showed that the deletion of one or two copies of HEB or E2A resulted in a proportional increase in the binding of the remaining E protein. This finding suggests that in the absence of adequate amounts of HEB:E2A heterodimers, HEB or E2A homodimers are capable of binding DNA (Barndt et al., 2000).

The ability of E proteins to functionally compensate for one another has rendered single gene knockouts only partially useful in efforts to understand E protein biology. These models likely underestimate the contribution of E protein function during lymphocyte development. Importantly, studies suggest limitations on the ability of E proteins to compensate for one another. This is supported by the fact that B cell development is not completely restored in E2A<sup>HEB</sup> mice (Zhuang et al., 1998). This discrepancy is also present in T cells, as evidenced by the surprising finding that E2A<sup>HEB</sup> T cells resemble those of the E2A knockout rather than WT counterparts (Y. Zhuang unpublished results). This result seems to suggest that while the kinetics and amplitude

of expression of E proteins may have a dramatic influence on the ability of one E protein to compensate for another, dimer-specific functions may exist.

To investigate the overlapping functions of HEB and E2A in T cell development, a dominant negative allele of HEB (HEB basic mutation or HEB<sup>bm</sup>) was created (Barndt et al., 2000). This variant is able to form dimers with E2A but cannot bind DNA due to the lack of the basic region required for DNA binding. As expected, HEB<sup>bm</sup> mice display a distinct and more severe phenotype than HEB or E2A single knockouts. These mice have a block at the DN3 stage of T cell development, a much more severe (15-100-fold) decrease in thymic cellularity, and are deficient in TCR $\beta$  V $_{\beta}$ -D $_{\beta}$ J $_{\beta}$  rearrangement (Figure 1.4A and Table 1.2).

Studies examining the effects of Id protein manipulation are also relevant to the investigation of E protein function and redundancy. Since these factors promiscuously antagonize all E protein function, compensation between different E proteins becomes less of a concern. However, in interpreting Id protein studies it should be noted that while Id proteins prefer E proteins as dimerization partners, these proteins have the potential to bind any bHLH protein. In addition, it is possible that Id proteins influence cellular functions independent of their ability to antagonize E protein activity (Ohtani et al., 2001; Yates et al., 1999).

In contrast to most E protein knockout studies, the disruption of individual Id genes generally causes little or no T cell developmental defects. Since other authors have found a cooperative role for Id proteins (Barone et al., 1994), this is likely the result of Id protein functional overlap such that the deletion of one may not sufficiently perturb E

protein activity. This redundancy is supported by findings that a defect in B cell proliferation caused by disruption of Id3 can be rescued by ectopic expression of Id1 (Pan et al., 1999).

### **1.3.5 E protein regulation of cell cycle**

Basic helix-loop-helix (bHLH) family E protein transcription factors have been implicated in the regulation of both differentiation and proliferation during T cell development. Because differentiation and cell growth are thought to be mutually exclusive events (Alema and TATO, 1987) and E proteins regulate both of these processes, this situates E proteins in a pivotal regulatory position during lymphocyte development. E proteins appear to have an inhibitory effect on cell cycle progression. In support of such a role, E2A has been shown to block the advancement of cells from the G<sub>1</sub>- to S-phase (Peverali et al., 1994). Also, the proportion of ISP stage thymocytes in S and G<sub>2</sub>/M phase is decreased in HEB<sup>ko</sup> mice (Barndt et al., 1999). Conversely, Id proteins act to promote G<sub>1</sub>- to S-phase transition (Barone et al., 1994; Hara et al., 1994; Sun et al., 1991). Accordingly, high levels of Id expression have been shown to favor proliferation and inhibit differentiation (Benezra et al., 1990). Further, Id expression has been shown to be downregulated during stages of differentiation in lymphocytes (Sun et al., 1991).

The mechanisms by which E proteins induce cell cycle arrest are incompletely understood and appear to be complex. E proteins have been implicated as being positive transcriptional regulators of the cell cycle inhibitor p21<sup>Cip1</sup> in non-T cells (Herblot et al., 2002; Pagliuca et al., 2000; Prabhu et al., 1997). Evidence for E proteins regulating

p21<sup>Cip1</sup> expression in T-lineage cells has also been reported, as E2A-deficient thymoma cells display an upregulation of p21<sup>Cip1</sup> when transduced with E47 (Schwartz et al., 2006). E proteins have also been shown to activate the expression of other CDKIs including p15<sup>Ink4b</sup> and p16<sup>Ink4a</sup> (Pagliuca et al., 2000). In addition to their role in regulating CDKI expression, E proteins function as positive transcriptional regulators of cyclins D2 and D3 (Zhao et al., 2001).

Additional evidence for the involvement of E proteins in regulating cell proliferation comes from studies showing that HEB<sup>bm</sup>, HEB-, and E2A-deficient mice display a high frequency of thymic derived T cell leukemia (Bain et al., 1997; Yan et al., 1997) (Y. Zhuang unpublished observations). In agreement with these findings, overexpression of Id proteins also results in lymphoma development (Kim et al., 1999).

## **1.4 Role of IL-7 in T cell development**

### **1.4.1 Interleukin-7 (IL-7)**

Cytokines are soluble low molecular weight proteins that are involved in a broad array of functions within the immune system including lymphocyte development, proliferation, and survival. The cytokine Interleukin-7 (IL-7) (previously called lymphopoietin1 and pre-B cell factor) was initially identified as a pre-B cell growth factor (Namen et al., 1988), but soon after was also found to induce T lymphocyte proliferation (Conlon et al., 1989; Grabstein et al., 1990; Morrissey et al., 1989). Following these early studies, other roles for IL-7 signaling during T cell development



were identified including T cell survival (Kim et al., 1998; von Freeden-Jeffry et al., 1997) and a role for IL-7 in TCR rearrangement (Candeias et al., 1997a; Candeias et al., 1997b; Durum et al., 1998; Ye et al., 2001). In addition, IL-7 is involved in maintaining the pool of mature and naïve peripheral T cells (reviewed by Jiang et al., 2005). Although a functional homolog of IL-7 exists called Thymic Stromal Lymphopoietin (TSLP), mice deficient in this protein have normal T cell function. This suggests that TSLP is not essential to lymphoid development (Jiang et al., 2005).

#### **1.4.2 IL-7 and IL-7R expression during T cell development**

IL-7 is a nonredundant cytokine essential for proper thymocyte development. During T cell development, thymocytes migrate through the different compartments of the thymus and are exposed to IL-7 produced by thymic stromal cells (Wiles et al., 1992). Specifically, IL-7 expression has been detected in the subcapsular region but not the cortex. Expression in the medullary regions is less clear, as some authors have detected expression while others have not (DeLuca and Clark, 2002; El Kassar et al., 2004). Because of this compartmentalization, exposure of developing thymocytes to physiological levels of IL-7 is thought to be locally restricted (Munitic et al., 2004). This geographic restriction serves to ensure appropriate developmentally regulated IL-7 signaling in thymocytes.

The functional IL-7 receptor (IL-7R) is composed of IL-7R $\alpha$  (CD127) and  $\gamma$ c (common gamma chain or CD132) heterodimer. While  $\gamma$ c is constitutively expressed on most hematopoietic cells, IL-7R $\alpha$  is expressed predominantly in lymphoid cells in a

stage-specific manner. IL-7R $\alpha$  expression begins at the common lymphoid progenitor (CLP) stage in the bone marrow (Schluns and Lefrancois, 2003). In developing T cells, IL-7R $\alpha$  expression is low in DN1 thymocytes, peaks at the DN2 stage, then rapidly declines in the DN3 and DN4 stages. Expression is completely extinguished by the ISP stage, which is thought to be essential for T cell progression, as signals from IL-7 inhibit DP thymocyte development by down-regulating the expression of transcription factors required for the ISP to DP transition (Yu et al., 2004). Although DP thymocytes do not express the IL-7R, it is re-expressed on cells undergoing positive selection (Munitic et al., 2004; Sudo et al., 1993; Van De Wiele et al., 2004).

### **1.4.3 IL-7R signaling**

Signaling through the IL-7R is complex and is mediated by a number of signaling pathways including the Jak/STAT and MAPK pathways, and PI3K (reviewed by Jiang et al., 2005). Heterodimerization of the IL-7R $\alpha$  and  $\gamma$  chains is required for activation of IL-7-mediated signaling events (Ziegler et al., 1995). Prior to dimerization, JAK3 is associated with the  $\gamma$  chain cytoplasmic domain and Jak1 is associated with the IL-7R $\alpha$  chain. Dimerization of the receptor allows transphosphorylation of Jak1 and Jak3 as well as phosphorylation of the IL-7R $\alpha$  chain. This receptor phosphorylation then attracts SH2-domain containing intracellular signaling molecules including STAT and Src family kinase members that reside in the cytoplasm. Once recruited, STATs are phosphorylated by Jak proteins. Upon phosphorylation, STATs dissociate with the receptor cytoplasmic tail and heterodimerize with other STAT members, which allows

their translocation to the nucleus and subsequent target gene transcription (Barata et al., 2005; Rawlings et al., 2004). A consequence of Jak/STAT activation is the activation of the receptor tyrosine kinase (RTK)/Ras/MAPK (mitogen-activated protein kinase) pathway. This can occur by Jak-mediated receptor tyrosine phosphorylation and subsequent recruitment of SH2-containing adaptor proteins from neighboring signaling pathways (Rawlings et al., 2004). Also recruited to the IL-7R $\alpha$  chain and phosphorylated (activated) is PI3K (Venkitaraman and Cowling, 1994).

The contribution of each signaling pathway to the anti-apoptotic and/or proliferation-inducing effects of IL-7 signaling has not been fully characterized. In addition, it appears that a degree of functional redundancy exists among the different pathways. For instance, Jak3 and STAT5 signaling have been shown to induce Bcl-2 gene expression (Eynon et al., 1999; Suzuki et al., 2000). PI3K induces the serine/threonine kinase Akt/PKB, which also influences cell survival by inactivating the pro-apoptotic protein Bad (Khaled et al., 2002; Li et al., 2004). In addition, AKT has been shown to target p27<sup>Kip1</sup> for degradation and thus acts to promote cell cycle progression (Fujita et al., 2002). Similarly, Ras/MAPK signaling has also been shown to downregulate p27<sup>Kip1</sup> expression in a variety of cell types (reviewed by Pruitt and Der, 2001). Such redundancy in the IL-7 signaling pathway provides the cell with several means to regulate the critical processes of proliferation and survival in the event a particular pathway is disrupted.

#### 1.4.4 Requirement for IL-7 in T cell development

IL-7 signaling functions during T cell development in a stage-specific manner. Dependency on IL-7 for thymocyte survival and proliferation does not begin until the DN2 stage of development. DN1 cells are unresponsive to IL-7 signaling (El Kassar et al., 2004). Once cells reach the CD44<sup>+</sup>CD25<sup>+</sup> DN2 stage IL-7 signals are required for survival and proliferation (Akashi et al., 1997; Kim et al., 1998; Peschon et al., 1994; von Freeden-Jeffry et al., 1997). In fact, IL-7 has been shown to be essential for the DN2-3 transition both *in vivo* and *in vitro* (Wang et al., 2006). However, this dependency on IL-7 is short-lived. Once cells reach the late DN3 stage, IL-7 is no longer required (Di Santo et al., 1999; Trop et al., 2001). At this stage, pre-TCR signaling takes over the responsibility of maintaining cell viability and promoting proliferation. Cells between the  $\beta$ -selection checkpoint and positive selection are unresponsive to IL-7 signaling and show normal proliferation rates following exposure to elevated levels of IL-7 (Van De Wiele et al., 2004). These findings contribute additional support to the notion that the DN to DP progression is independent of IL-7 and relies only on pre-TCR-mediated signaling (Balciunaite et al., 2005; El Kassar et al., 2004). Importantly, the role of IL-7 in promoting cell survival and/or proliferation may be stage-specific in that it may promote one process vs. the other depending on the stage of development. For example, DN2 cells proliferate *in vivo*, while DN3 are arrested in cell cycle. While this correlates with the finding that DN2 cells are the main population of cells responsive to IL-7 (El Kassar et al., 2004), DN3 cells die in absence of IL-7 (Kim et al., 1998).

A variety of genetic models have been utilized to investigate the effects of disruption of IL-7 signaling on lymphocyte development. These models include IL-7<sup>-/-</sup> mice (von Freeden-Jeffry et al., 1995), IL-7R $\alpha$ <sup>-/-</sup> mice (Maki et al., 1996; Peschon et al., 1994), and  $\gamma$ c<sup>-/-</sup> mice (Cao et al., 1995; DiSanto et al., 1995). Regardless of the specific model, the common finding of all these knockout studies is a block at the DN stage of thymocyte development in the absence of functional IL-7 signaling.

IL-7 knockout mice exhibit a 20-fold decrease in the size of the thymus and a severe reduction in peripheral T cell numbers (von Freeden-Jeffry et al., 1995). Disruption of the  $\gamma$ c results in a similar phenotype (DiSanto et al., 1995). Mice deficient in the IL-7R $\alpha$  chain exhibit a more severe phenotype than IL-7 knockout mice. IL-7R-deficient mice typically demonstrate a strong block at the DN stage of thymocyte development and have a thymic cellularity <1% of WT controls. The block in T cell differentiation in IL-7 and IL-7R deficient mice is not complete and some peripheral T cells are present (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). However, the few mature T cells that develop in these mice are functionally impaired in their ability to respond to antigenic and mitogenic stimulation (Maraskovsky et al., 1996).

A number of IL-7 transgenic mice have been employed to investigate the role of IL-7 in T cell development. Results from these studies are consistent with an essential role for IL-7 signaling in promoting thymocyte development, proliferation, and survival. In addition, these studies indicate that excessive IL-7 signaling can contribute to lymphoproliferative disorders (El Kassar et al., 2004; Mertsching et al., 1995; Munitic et al., 2004; Rich et al., 1993; Samaridis et al., 1991; Williams et al., 1997). Soluble IL-7,

as well as blocking antibodies to IL-7 or IL-7R have also been employed in an attempt to more fully understand the role of IL-7 in lymphopoiesis. *In vivo* administration of IL-7 to normal mice results in a transient increase in peripheral T cell numbers (Morrissey et al., 1991). In contrast, the use of blocking antibodies to IL-7 or IL-7R results in the depletion of post-DN2 thymocytes (Grabstein et al., 1993; Sudo et al., 1993). In summary, these models all point to a critical role for IL-7 in early thymocyte development by regulating proliferation and survival.

#### **1.4.5 Regulation of cell survival and proliferation by IL-7**

IL-7 has been implicated as a positive regulator of cellular proliferation and survival. Importantly, these functions of IL-7 signaling appear independent. Promotion of cell survival by IL-7 is accomplished by regulating the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family members (El Kassar et al., 2004; von Freeden-Jeffry et al., 1997). IL-7 signaling stimulates the expression of anti-apoptotic factors such as Bcl-2 and Mcl-1 and inhibits the expression of pro-apoptotic factors Bad, Bax, and Bim (reviewed by Jiang et al., 2005). In addition to the well-characterized role for IL-7 in regulating cell survival, an independent but less understood function of IL-7 is to promote cell proliferation. IL-7 induces proliferation of DN and mature SP thymocytes (Okazaki et al., 1989). Mice deficient in IL-7 display an increased percentage of thymocytes in G<sub>0</sub>/G<sub>1</sub> phase and a corresponding decrease in the percentage in S and G<sub>2</sub>/M phases (von Freeden-Jeffry et al., 1997). This finding suggests that IL-7 signaling may function to promote the G<sub>1</sub> to S phase transition, a step that is regulated by cyclin D/CDK4, cyclin

D/CDK6, and cyclin E/CDK2 complexes. However, no increase in cyclin D or cyclin E expression has been reported in cells responding to IL-7, suggesting another mechanism must be responsible for the induction of proliferation by IL-7 signaling (unpublished observations cited by Jiang et al., 2005). Indeed, it appears the post-translational downregulation of the CDKI p27<sup>Kip1</sup> is the primary mechanism responsible for IL-7-mediated proliferation (Barata et al., 2001; Hofmeister et al., 1999; Li et al., 2006). Importantly, although this may be the principle means by which IL-7 induces proliferation, other secondary mechanisms exist. For instance, IL-7 can induce the expression of *c-myc* (Crawley et al., 1996; Morrow et al., 1992), which can stimulate cyclin D-CDK4/6 activity and therefore promote cell proliferation (Mateyak et al., 1999). Also, IL-7 can maintain the Cdc25A phosphatase in an active (dephosphorylated) state, which also promotes CDK2 activation and thereby promotes the G<sub>1</sub> to S phase transition (Khaled et al., 2005).

Together, these results indicate a role for IL-7 in the regulation of both cell survival and cell proliferation. The finding that Bcl-2 can rescue  $\alpha\beta$  T lymphocyte development in IL-7R<sup>-/-</sup> mice suggests that the anti-apoptotic role of IL-7 is its main function during thymocyte development (Maraskovsky et al., 1997). However, IL-7 is required for T cell homeostatic proliferation, and Bcl-2 overexpression cannot substitute for this function (Tan et al., 2001). Similarly, a thymocyte cell line transfected with Bcl-2 is able to survive in the absence of IL-7, but is unable to proliferate (Li et al., 2006). These results suggest that while the cell survival function of IL-7 may be necessary for

proper T cell development, it is not sufficient for the induction of proliferation, which is also a crucial event during thymocyte development.



**CHAPTER 2**  
**MATERIALS AND METHODS**

## 2.1 Construction of the HEB-loxP targeting construct

Genomic HEB sequence encoding the bHLH exon was cloned into pSK(+) immediately upstream of a floxed PGK-Neomycin positive selection cassette. A partial digest using BamHI was performed to insert an additional loxP site 5' of the bHLH exon. A 1.1kb fragment was then cloned into a SpeI site upstream of the floxed bHLH domain to serve as the short arm for homologous recombination. The construct was excised using BglII and inserted into a shuttle vector containing a 7kb long arm corresponding to the genomic sequence downstream of the bHLH exon, along with a PGK-thymidine kinase (TK) negative selection cassette. The complete targeting construct was linearized and transfected into cultured embryonic stem (ES) cells. These cells were grown under dual selection conditions using G418 and gancyclovir and surviving clones were screened by PCR for homologous recombination using a primer upstream of the short arm (genomic dnteam: 5'-CTTAGGACATGCGTTCATAATAAC-3') and a primer that partially hybridizes to the loxP site upstream of the bHLH region (lox/bHLH upstream: 5'-GTTATTCAGACTGGATCTAATAAC-3'). Following the identification of successful recombinants (approximately 5% of all clones (14 out of 270)) and subsequent karyotyping, the ES cell clones were transiently transfected *in vitro* with a Cre-recombinase expression vector and screened by PCR for the floxed HEB allele (PGK-Neo deleted) and SpeI digest. Although we were unable to detect bHLH deletion alone due to PCR design, percentages for total deletion (both the bHLH region + PGK-Neo removed) and PGK-Neo deletion alone were 11.5% and 1%, respectively. Primers used for detection of Cre-deleted products were genomic dnteam (see above for sequence)

and a primer that hybridizes with the long arm of the targeting construct (upstream long arm: 5'-GCCAGAGGGTGGGAAGCTCAGG-3'). These clones were then injected into blastocysts and implanted into pseudo-pregnant foster recipient mice to generate chimeras. Following chimera matings, F1 offspring containing the heterozygous HEB<sup>flx/+</sup> allele were produced. HEB<sup>flx/+</sup> mice were then crossed with E2A<sup>flx/+</sup> lck-Cre<sup>+</sup> mice (Pan et al., 2002) to establish HEB<sup>flx/flx</sup> E2A<sup>flx/flx</sup> Cre<sup>+</sup> (DKO) and HEB<sup>flx/flx</sup> Cre<sup>+</sup> (SKO) mice. Viability and fertility of the HEB<sup>flx/flx</sup> Cre<sup>+</sup> as well as E2A<sup>flx/flx</sup> Cre<sup>+</sup> mice are similar to those of WT mice.

## 2.2 PCR genotyping

The presence of floxed or WT HEB and E2A alleles as well as the Cre transgene was detected by PCR. HEB was genotyped using JW1 and JW2; E2A was genotyped using three primers: YZ-104, YZ-150, and YZ-164; and the presence of Cre detected using Lck-P and Mx3. Primer sequences and PCR conditions are listed in Tables 2.1A and 2.1B, respectively. Size of amplification products are: HEB<sup>flx</sup> 1.3kb; HEB<sup>WT</sup> 1.05kb; E2A<sup>flx</sup> 2.0kb; E2A<sup>WT</sup> 1.8kb; Cre 800bp.

## 2.3 PCR detection of Cre-mediated deletion

To amplify floxed and deleted bands for HEB or E2A, genomic DNA was harvested from sorted DN2-4 thymocytes or Day 5 *ex vivo* cultured DKO cells and subject to 'touchdown' PCR (Jackson et al., 2005) using JW1 and JW2 primers for HEB and three primers for E2A: E2A<sup>flx</sup> fw, PGKNeo fw, and YZ-198. Primer sequences and

**Table 2.1 Primers and PCR conditions for genotyping of HEB and E2A WT and floxed alleles.** (A) Detection of HEB was accomplished using JW1 and JW2. E2A was genotyped using 3 primers: YZ-104, YZ-150, and YZ-164. Cre transgene detection was accomplished using LckP and Mx3. (B) PCR conditions for genotyping.

**A**

Genotyping	Primers	Sequence (5'-3')
HEB	JW1 JW2	CTGGGACAGAAGTTCAGCACTTAGTAC CATTCCTATACATCAGCTTCTTGGACG
E2A	YZ-104 YZ-150 YZ-164	ATGTGTGGTGGCCCACACTTGT ACATGGCTGAATATCGACGGT AAGAACGAGGCCTTCCGTGTC
Cre	Lck-P Mx3	GCAGGAAGTGGGTAAGTACTAGACTAA TCTCCACCGTCAGTACGTGAGATATC

**B**

Step	Temp (deg C)	Duration (min)	Phase
1	93	1.5	Initial denaturation
2	93	0.5	Denaturation
3	57	0.5	Annealing
4	65	3	Extension
5	Go to Step 2 39x		
6	End		

PCR conditions are listed in Tables 2.2A and 2.2B, respectively. Toe DNA from a HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>-</sup> mouse was used as a negative control. To quantify deletion, a standard curve was generated by mixing known numbers of HEB<sup>-/-</sup> E2A<sup>-/-</sup> and HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> pre-B cells together in the following ratios: 100% del, 90% del: 10% flox, 75% del: 25% flox, 50% del: 50% flox, 25% del: 75% flox, 10% del: 90% flox, 100% flox (Lazorchak et al., 2006).

## 2.4 PCR analysis of TCR $\beta$ V(D)J rearrangement

Total thymus genomic DNA was harvested from LAT<sup>-/-</sup>, Rag2<sup>-/-</sup>, HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>-</sup>, and HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>+</sup> mice or sorted Day 7 *ex vivo* cultured DKO thymocytes and subjected to ‘touchdown’ PCR (Jackson et al., 2005). Primers were designed using a consensus sequence to detect rearrangements using the V $\beta$ 5 and V $\beta$ 8 gene families. 5’ primers for both V $\beta$  gene families were used in conjunction with J $\beta$ 2.7-3’ as the 3’ primer. EF1 $\alpha$  was amplified as a loading control using YZ-95 and YZ-96. Primer sequences and PCR conditions are listed in Tables 2.3A and 2.3B, respectively. Platinum Taq polymerase was used for PCR amplification as recommended by the manufacturer (Invitrogen).

## 2.5 Flow cytometry analysis

Single-cell suspensions were harvested from thymus, resuspended in 1X PBS/5% bovine calf serum, and kept on ice throughout the analysis. Cells were stained on ice for 30 minutes in the dark using the appropriate mAbs and scored using a FACSCalibur (Becton Dickinson). FloJo software (Tree Star Inc.) was used for data analysis. All

**Table 2.2 Primers and touchdown PCR conditions for detection of HEB and E2A floxed and deleted alleles.** (A) JW1 and JW2 primers were used for HEB; E2A<sup>flox</sup> fw, PGKNeo fw, and YZ-198 primers for E2A. (B) Touchdown PCR conditions for detection of floxed vs. deleted alleles of HEB and E2A.

**A**

Target	Primers	Sequence (5'-3')
HEB	JW1	CTGGGACAGAAGTTCAGCACTTAGTAC
	JW2	CATTCCTATACATCAGCTTCTTGGACG
E2A	E2A <sup>lox</sup> fw	CTGCACTCCGAATTGTGCCTG
	PGKNeo fw	GCCCATTCGACCACCAAGCG
	YZ-198	GATCCTCGTCTTCATTGGTACTG

**B**

Step	Temp (deg C)	Duration (min)	Phase
1	95	5	Initial denaturation
2	95	0.5	Denaturation
3	65	0.5	Annealing
4	72	2	Extension
5	Go to Step 2 4x		
6	95	0.5	
7	62	0.5	
8	72	2	
9	Go to Step 6 4x		
10	95	0.5	
11	60	0.5	
12	72	2	
13	Go to Step 10 4x		
14	95	0.5	
15	56	0.5	
16	72	2	
17	Go to Step 14 26x		
18	72	5	Final Extension
19	End		



**Table 2.3 Primers and touchdown PCR conditions for detection of  $V_{\beta}$ - $D_{\beta}$ - $J_{\beta}$  recombination.** (A) Consensus primers were used to detect rearrangements using the  $V_{\beta}5$  and  $V_{\beta}8$  families. The same  $J_{\beta}2.7$  primer was used with both  $V_{\beta}$  primers. EF1 $\alpha$  was used as a loading control. (B) Touchdown PCR conditions for detection of recombination.

**A**

Target	Primers	Sequence
V $\beta$ 5 gene family	V $\beta$ 5-5' consensus J $\beta$ 2.7-3'	CCCAGCAGATTCTCAGTCCAACAG TGAGAGCTGTCTCCTACTATGGATT
V $\beta$ 8 gene family	V $\beta$ 8-5' consensus J $\beta$ 2.7-3'	GCATGGGCTGAGGCTGATCCATTA TGAGAGCTGTCTCCTACTATGGATT
EF1 $\alpha$	YZ-95 YZ-96	AGTTTGAGAAGGAGGCTGCT CAACAATCAGGACAGCACAGTC

**B**

Step	Temp (deg C)	Duration (min)	Phase
1	95	5	Initial denaturation
2	95	1	Denaturation
3	65	1	Annealing
4	72	3	Extension
5	Go to Step 2 4x		
6	95	1	
7	62	1	
8	72	3	
9	Go to Step 6 4x		
10	95	1	
11	60	1	
12	72	3	
13	Go to Step 10 4x		
14	95	1	
15	58	1	
16	72	3	
17	Go to Step 14 20x		
18	72	5	Final Extension
19	End		

antibodies used for staining were purchased from BD-PharMingen, eBioscience, or Caltag. BrdU staining was performed using a BrdU Flow Kit (BD Pharmingen) as per manufacturer instructions where mice were injected IP with 1mg BrdU and sacrificed 4 hours later.

## 2.6 Cell sorting

To purify CD4<sup>-</sup>CD8<sup>-</sup> (DN) populations, thymocytes were first stained with anti-CD4-PE/Cy5 and anti-CD8a-PE/Cy5 antibodies. CD4<sup>+</sup> and CD8<sup>+</sup> cells were depleted with Dynabeads conjugated to sheep anti-rat IgG (Invitrogen). For some experiments biotin-conjugated antibodies were used followed by anti-biotin Dynabead depletion. No differences were detected between the two methods. DN enriched thymocytes were further stained with anti-CD44, anti-Thy-1.2, and anti-CD25. DN subpopulations were defined and sorted as CD4<sup>-</sup>CD8<sup>-</sup>CD44<sup>+</sup>CD25<sup>-</sup> (DN1), CD4<sup>-</sup>CD8<sup>-</sup>CD44<sup>+</sup>CD25<sup>+</sup> (DN2), CD4<sup>-</sup>CD8<sup>-</sup>CD44<sup>-</sup>CD25<sup>+</sup> (DN3), and CD4<sup>-</sup>CD8<sup>-</sup>CD44<sup>-</sup>CD25<sup>-</sup>Thy-1.2<sup>+</sup> (DN4). For purification of HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>+</sup> DN populations, thymocytes were directly stained with the above antibodies for sorting without any magnetic bead separation. Dead cells were excluded from sorting as positively stained cells with propidium iodide. FACS sorting were performed on a FACSVantage with a DiVa option equipped with 488 nm argon, 599 nm dye, and 408 nm krypton lasers (BD Bioscience Flow Cytometry Systems).

## 2.7 Real-time PCR

Messenger RNA (mRNA) was harvested from sorted DN3 and DN4 thymocytes (see above) or Day 5 *ex vivo* cultured DKO or LAT cells using TRI reagent as recommended (Sigma) and reverse transcribed to cDNA using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase kit per manufacturer instructions (Invitrogen). cDNA was then subjected to SYBR Green real-time PCR (Roche). Relative units for each target were calculated using EF1 $\alpha$  as a standard. Primer sequences are listed in Table 2.4.

## 2.8 Ex vivo proliferation assay

For cell enumeration experiments, total DN cell culture was performed with 2.4 to 2.7 x 10<sup>5</sup> cells per 1mL RPMI culture medium (Gibco) containing 10% FBS, 10ng/mL IL-7, 1x  $\beta$ -Mercaptoethanol ( $\beta$ -ME) (55uM), and 1x Penicillin/Streptomycin in a 24-well plate. Cells from single wells were harvested at specified time points for FACS analysis and enumerated on a hemacytometer. Trypan blue staining was used to exclude dead cells. [<sup>3</sup>H]-thymidine incorporation assays on sorted DN cells were done as follows: WT, p18<sup>Ink4c<sup>-/-</sup></sup>, and p21<sup>Cip1<sup>-/-</sup></sup> thymocytes were first depleted of CD4<sup>+</sup> and CD8<sup>+</sup> cells using Dynabeads as described above. For total DN thymocyte cultures, 1x10<sup>5</sup> purified DN thymocytes were cultured in triplicate wells of a 96 well plate in 100mL Iscove's Modified Dulbecco's Medium containing 5% FCS, with or without IL-7 (10ng/mL, R&D) for 48 hours. [<sup>3</sup>H]-thymidine (1 mCi) was added into the culture 6 hrs before

**Figure 2.4 Primers used in real-time PCR analysis.**

Target	Primers	Sequence
Pre-T alpha	pTa fw pTa rev	CCATCACACTGCTGGTAGATGGAA GCAGAAGCAGTTTGAAGAGGAGCA
p18Ink4C	p18 F1 p18 R1	ACACTGTACAGGCTTTGCTGGAGT CACATTGCAGGCTGTGTGCTTCAT
p21Cip1	p21 fw p21 rev	TTGTCGCTGTCTTGCACTCTGGT AGACCAATCTGCGCTTGGAGTGAT
p27Kip1	p27 fw p27 rev	CGTGAGAGTGTCTAACGGGAGCC GCTTCTTGGGCGTCTGCTCCA
cyclin D3	cyclin D3 fw cyclin D3 rev	ATACTGGATGCTGGAGGTGTGTGA AGCGATCCAGGTAGTTCATAGCCA
cyclin E	cyclin E fw cyclin E rev	TGAGTTCCAAGCCCAAGTCCTG CCAAACCTCTTCTCTAATGCCCC
cyclin B1	cyclin B fw cyclin B rev	CGGAGGAACGGCTGTTAGTTTTAG CCCTTTTCAGAGGCACTGTTGC
IL-7Ra	IL-7R fw IL-7R rev	ACTTCAAAGGCTTCTGGAGCGAGT ATGGTGACACTTGGCAAGACAGGA
Jak3	Jak3 S1 fw Jak3 S1 rev	GCGCAAAGATTTGACCAGTGCCAT ACTCTCCCTGCTCCTTCATGCTAA
STAT5	STAT5 S3 fw STAT5 S3 rev	TGGTTCGCTGTATCCGTCACATTC TGATCTGAAGGTGCTTCTGGGACA
FOXO1	FOXO1 S5 fw FOXO1 S5 rev	AAGAGCGTGCCCTACTTCAAGGAT TCCAGTTCCTTCATTCTGCACTCG
GSK3b	GSK3b S2 fw GSK3b S2 rev	TCCGAGGAGAGCCCAATGTTTCAT TTCCACCAACTGATCCACACCACT
Pten	Pten S3 fw Pten S3 rev	AATTCCCAGTCAGAGGCGCTATGT TCTTCACCTTTAGCTGGCAGACCA
Men1	Men1 fw Men1 rev	TCTTCAGCTTCATCACAGGCACCA AAGCATGATCTTCAGACAGGGCCA

harvesting. Cells were harvested with an auto cell harvester (Harvester 96<sup>®</sup>, TOMTEC) onto a glass filter. Radioactivity was determined by a liquid scintillation counter (1450 LSC & Luminescence Counter, PerkinElmer). Sorted DN2 and DN3 cultures were done similarly, except  $1 \times 10^4$  cells were cultured for 7 days and [<sup>3</sup>H]-thymidine was added into the culture 14-16 hrs before harvesting. Fresh IL-7 was added at day 3 and 6 of culture.

## **2.9 Dual–Luciferase reporter assay**

Jurkat, EL4, or p5424 cells (a pre-T p53<sup>-/-</sup> RAG1<sup>-/-</sup> cell line) were maintained in RPMI (Gibco) containing 10% FBS, 55uM β-ME, and 1x Penicillin/Streptomycin. Immediately prior to transfection, cells were placed in Opti-MEM reduced serum medium (Gibco). Cells were transfected with lipofectamine 2000 as recommended by the manufacturer (Invitrogen). Cells received 0.5ug of a E47 expression vector in combination with 0.5 ug of the appropriate pGL2 vector containing the p18 5' promoter fragments which were used to drive luciferase expression (a kind gift from Dr. Yue Xiong). All samples also received 25ng of pRL renilla luciferase vector to normalize for transfection efficiency.  $1 \times 10^6$  cells were transfected and were analyzed for luciferase expression 24 hrs post-transfection on DLR luminometer (Promega).

## **2.10 OP9/OP9-DL1 stromal cultures**

OP9 or OP9-DL1 stromal cells were plated in a 24-well plate 24h before DKO coculture in MEM Alpha (Gibco) containing L-glutamine, 20% FBS, 1x Penicillin/Streptomycin, and 1x sodium pyruvate (1mM). At the time of addition of

1x10<sup>6</sup> DKO thymocytes, stromal cells were 70-80% confluent and the media was replaced with Iscove's Modified Dulbecco's Meduim (Gibco) containaing L-glutamine, 5% FBS, 1x Sodium pyruvate (1mM), 1x Penicillin/Streptomycin, 1x β-ME (55um). Cells were harvested at indicated time periods and analyzed by FACS staining using VCAM1<sup>+</sup> gating to eliminate stromal cell contamination.



## **CHAPTER 3**

### **E PROTEINS REGULATE THYMOCYTE DIFFERENTIATION**

### 3.1 Introduction

Previous studies clearly implicate a crucial role for E proteins during T cell development. Disruption of E protein function has a wide range of developmental consequences depending on the specific E protein and model system utilized. Germline E2A deficient mice display a partial block at the DN1 stage of thymocyte development and a reduction in thymic cellularity (Bain et al., 1997). In contrast, T cell-specific conditional deletion of E2A using an *Lck-Cre* transgene does not result in any major defect in thymocyte development (Pan et al., 2002). Also, while HEB knockout mice show a block at the immature single-positive (ISP) stage and a 5-10-fold decrease in thymocyte number (Barndt et al., 1999), HEB<sup>bm</sup> mice display a DN3 block and much more severe decrease in thymic cellularity (Barndt et al., 2000).

A considerable obstacle to the direct elucidation of E protein function during lymphocyte development is the existence of functional compensation among different E proteins. Such functional redundancy has plagued earlier studies and is phenotypically manifested by the finding that single E protein deletions only have limited developmental defects. This overlap, in addition to the pleiotropic effect of germline gene disruption and corresponding high rates of neonatal lethality has obscured the role of E proteins during T cell development.

To address these issues and facilitate the investigation of E protein function in T cell development, we have created a T cell-specific E2A and HEB double conditional knockout (DKO) mouse employing a Cre-recombinase system. This model is particularly useful because HEB:E2A heterodimers are thought to be the main form of

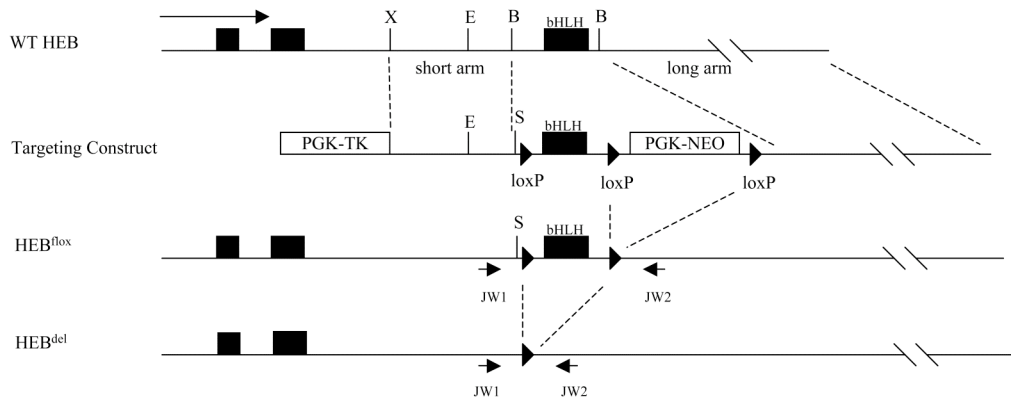
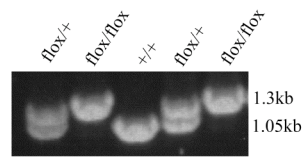
functional E proteins in T cells and thus this knockout should effectively abrogate any concerns of functional compensation between E2A and HEB proteins.

## **3.2 Results**

### **3.2.1 Construction of a HEB floxed allele**

The development of a HEB conditional knockout was undertaken for the parallel aims of investigating the lineage- and stage-specific function of HEB in T cell development, and for the creation of a HEB-E2A double conditional knockout. To these ends, we employed a loxP-Cre conditional knockout model. The conditional HEB knockout targeting construct was created by flanking both exon 18 of genomic HEB and the PGK-Neo selection cassette with loxP sites (triple loxP system) (Figure 3.1A). Exon 18 of HEB encodes the basic-helix-loop-helix (bHLH) domain, which is indispensable for DNA-binding and dimerization (Murre et al., 1989a). Targeted ES cell clones were transiently transfected with a Cre-recombinase expression vector and screened for those clones in which the floxed bHLH region remained but the PGK-Neo cassette had been deleted. The ES cell clones carrying the HEB floxed allele (HEB<sup>flox</sup>) were introduced into a mouse embryo for germline transmission. Subsequent breeding revealed that the floxed allele segregated with the same ratio as the wild type allele (WT) (Figure 3.1B) and that HEB<sup>flox/flox</sup> mice were phenotypically indistinguishable from WT littermates. Importantly, HEB<sup>flox/flox</sup> mice did not show any associated postnatal lethality and were similar to WT controls in terms of viability and fertility.

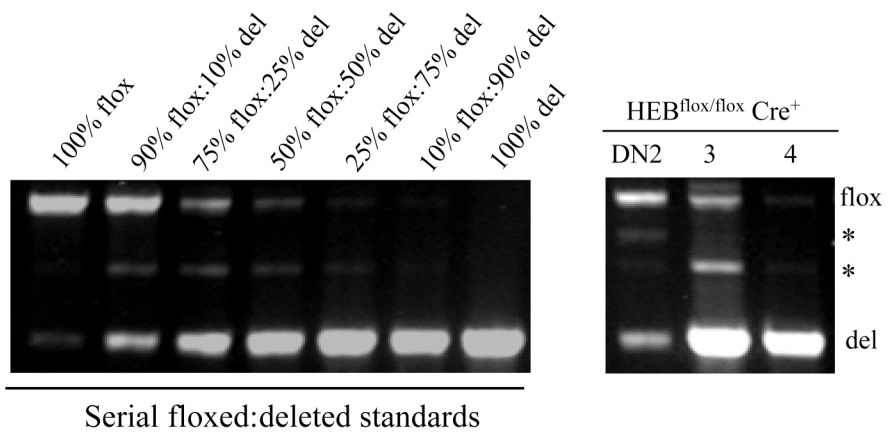
**Figure 3.1 Generation of a HEB<sup>fllox</sup> allele.** (A) The targeting strategy for HEB<sup>fllox</sup> mice. Shown are partial representations of the HEB genomic locus, the targeting construct, the floxed HEB (HEB<sup>fllox</sup>) allele, and the deleted HEB (HEB<sup>del</sup>) allele. Restriction enzyme site designations are as follows: B= BamHI, E=EcoRI, S=SpeI, and X=XhoI. Exons are depicted by black boxes and solid triangles represent loxP sites. The arrow above the WT HEB diagram indicates the direction of transcription. The primers JW1 and JW2 used for genotyping PCR are shown. (B) HEB<sup>fllox</sup> genotyping PCR. Mouse toe DNA was harvested and the primers JW1 and JW2 were used to detect WT and HEB floxed alleles, yielding 1.05kb and 1.3kb fragments, respectively.

**A****B**

### 3.2.2 Conditional deletion of HEB results in a block at the immature single-positive (ISP) stage of T cell development

Mice carrying the HEB<sup>flox</sup> allele were crossed to *lck*-Cre transgenic mice. This transgene was previously utilized in our lab for the construction of E2A conditional knockout mice (Pan et al., 2002) and provides T-lineage-specific expression of Cre recombinase under the control of the *Lck* gene proximal promoter. To examine the expression pattern of Cre and the efficiency of HEB deletion in developing thymocytes, we assessed the presence of floxed and deleted bands in DN2-4 sorted thymocytes from HEB<sup>flox/flox</sup> Cre<sup>+</sup> mice. Serial dilutions of control DNA with predetermined ratios of floxed to deleted ranging from 100% floxed to 100% deleted were used to estimate the amount of deletion in HEB<sup>flox/flox</sup> Cre<sup>+</sup> mice (Lazorchak et al., 2006). PCR analysis shows that deletion of HEB occurs during the double-negative stage of thymocyte development, starting as early as the DN2 stage and persisting through the DN4 subset, where deletion appears to be at least 75% complete (Figure 3.2). Flow cytometric analysis of total thymocytes from HEB<sup>flox/flox</sup> Cre<sup>+</sup> mice showed an accumulation of CD8 single-positive cells (Figure 3.3A). CD5 expression was then evaluated as a marker to differentiate between mature CD8<sup>+</sup> and immature single-positive (ISP) CD8<sup>+</sup> cells, as the latter cell type have lower CD5 expression levels than mature CD8<sup>+</sup> cells (Barndt et al., 1999). As such, CD5 staining revealed that the block was at the ISP stage of development. DP cells also show two distinct peaks for CD5 expression, a finding that is consistent with the HEB germline knockout (Barndt et al., 1999). A 3-fold average

**Figure 3.2 *Lck*-Cre-mediated deletion of HEB occurs in the DN compartment.** The presence of floxed vs. deleted HEB alleles in sorted DN2-4 thymocytes from HEB<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> Cre<sup>+</sup> single knockout (SKO) mice. Genomic DNA was harvested from DN2-4 thymocytes and PCR was performed with JW1 and JW2 primers. A HEB serial dilution PCR with known ratios of floxed vs. deleted alleles is shown for reference to estimate the efficiency of deletion. Non-specific products are marked by an asterisk.





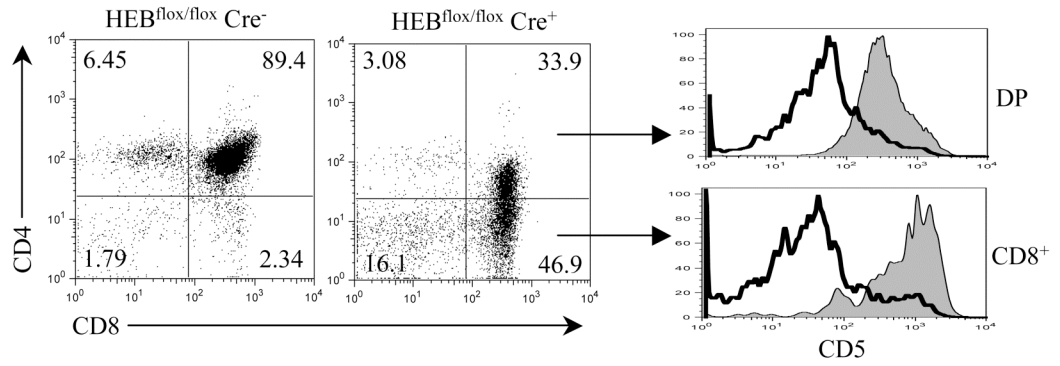
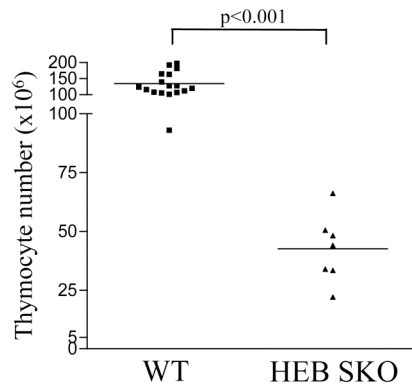
reduction in thymocyte numbers was detected in HEB<sup>flx/flx</sup> Cre<sup>+</sup> mice compared with WT or HEB<sup>flx/flx</sup> Cre<sup>-</sup> controls ( $39.9 \pm 15.4 \times 10^6$  vs.  $133 \pm 32.6 \times 10^6$ , respectively) (Figure 3.3B). Importantly, this phenotype closely resembles that of conventional HEB knockout mice, which demonstrate a block at the immature single-positive (ISP) stage of development and a 5-10-fold reduction in thymocyte numbers (Barndt et al., 1999). This finding confirms the importance of HEB in T cell differentiation and suggests that the conditional deletion of HEB is either complete or at least to a sufficient degree such that its function is abrogated by the ISP stage of thymocyte development.

### **3.2.3 Conditional deletion of both HEB and E2A results in a block at the DN stage of thymocyte development**

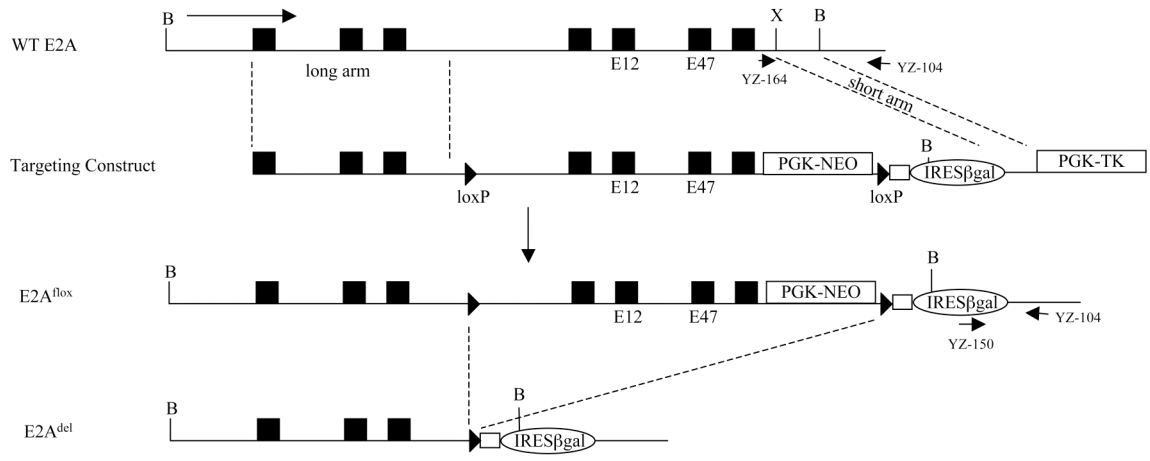
Functional compensation between HEB and E2A has been well documented in early studies using HEB and E2A single gene knockouts. Therefore, we attempted to more clearly investigate the function of these E proteins in T cell development by creating a HEB and E2A double conditional knockout. This model is valuable in that it eliminates the compensation issues that have hindered previous studies. HEB<sup>flx/flx</sup> mice were mated to previously generated E2A<sup>flx/flx</sup> *Lck-Cre* (Cre<sup>+</sup>) mice (Pan et al., 2002) to obtain HEB<sup>flx/flx</sup> E2A<sup>flx/flx</sup> Cre<sup>+</sup> mice. The targeting strategy used for generation of the E2A conditional knockout is illustrated in Figure 3.4.

Thymocyte development in conditional HEB and E2A double-knockout (DKO) mice is blocked at the double-negative (DN) stage of differentiation (Figure 3.5A). Examination of DN1-4 cell subsets using surface CD44 and CD25 staining reveals a

**Figure 3.3 Conditional deletion of HEB results in a block at the ISP stage of thymocyte development.** (A) Deletion of HEB results in a block at the immature single positive (ISP) stage of thymocyte development. Total thymocytes were harvested from HEB<sup>flox/flox</sup> Cre<sup>-</sup> and HEB<sup>flox/flox</sup> Cre<sup>+</sup> mice and subject to flow cytometric analysis after staining with CD8-FITC, CD5-PE, 7AAD, Mac-1-TriColor (TC), Gr-1-TC, B220-TC, and CD4-APC. Following gating on forward and side scatter, 7AAD and Mac-1, Gr-1, and B220 staining was used to exclude dead cells and non-T cells from the analysis, respectively. Percentage of cells in each quadrant is indicated. CD5 histograms are shown to differentiate between DP and ISP CD5<sup>low</sup>CD8<sup>+</sup> and mature CD5<sup>hi</sup>CD8<sup>+</sup> T cells. Arrows indicate quadrant origin for CD5 plots. Shaded areas represent HEB<sup>flox/flox</sup> Cre<sup>-</sup> thymocytes, solid lines without shading represent HEB<sup>flox/flox</sup> Cre<sup>+</sup> thymocytes. (B) Decreased thymic cellularity in HEB conditional knockout mice. Total thymocytes from WT (n=17) and HEB<sup>flox/flox</sup> Cre<sup>+</sup> single conditional knockout (SKO) (n=8) were harvested and counted using a Coulter Counter as per manufacturer recommendations. T-test P values between WT and HEB conditional knockout cell numbers are indicated.

**A****B**

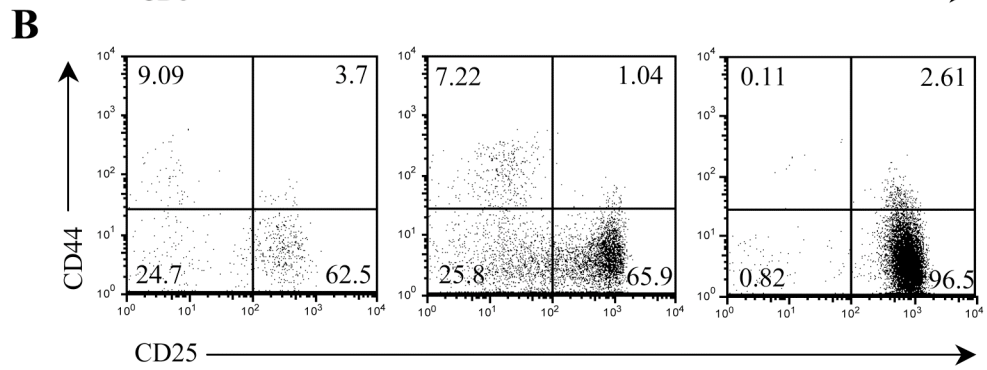
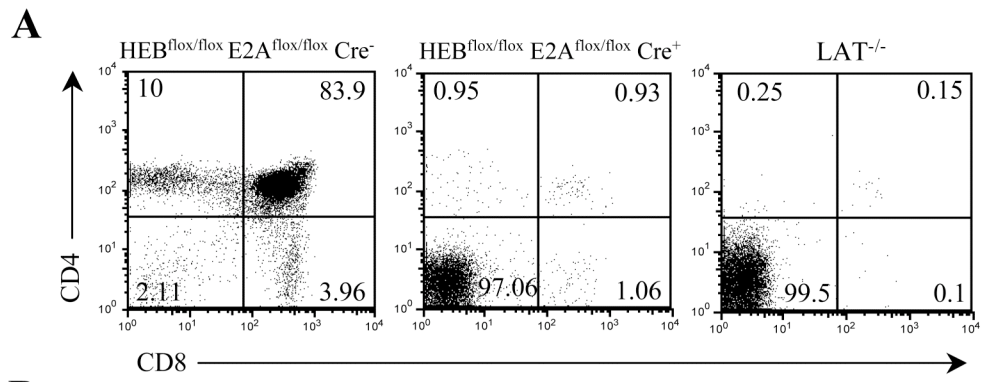
**Figure 3.4 Generation of an E2A<sup>fllox</sup> allele.** Shown are partial representations of the E2A genomic locus, the targeting construct, the E2A<sup>fllox</sup> allele, and the E2A<sup>del</sup> allele. Restriction enzyme site designations are as follows: B= BamHI and X=XbaI. Exons are depicted by black boxes and solid triangles represent loxP sites. The primers YZ-104, YZ-150, and YZ-164 used for genotyping PCR are shown. Adapted from Pan et al., 2002.



relatively normal distribution of cells throughout the DN stages of development (Figure 3.5B). This is in contrast to E2A or HEB single knockouts, which manifest partial blocks at the DN1 and ISP stages, respectively (Bain et al., 1997; Barndt et al., 1999; Barndt and Zhuang, 1999). LAT deficient thymocytes were used as a control, as these cells are also blocked at the DN stage of development. In contrast to the DKO however, LAT<sup>-/-</sup> thymocytes are arrested at the DN3 stage (Zhang et al., 1999).

Very few double-positive cells are observed in all DKO mice examined (< 1% of wild type). Total thymic cellularity is significantly reduced (average 20-fold) in HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>+</sup> mice in comparison to WT or Cre-negative controls ( $6.7 \pm 3.1 \times 10^6$  vs.  $134.4 \pm 33.4 \times 10^6$ , respectively) and almost 10-fold compared with the HEB single conditional knockout (Figure 3.6A). FACS analysis of DKO and WT thymi consistently reveals that the average proportion of DN cells is approximately 95% and 3% of the total thymocyte pool, respectively (data not shown). Therefore, although total thymic cellularity is decreased in DKO mice, there is approximately 50% greater numbers of DN thymocytes in DKO mice than WT controls (Figure 3.6B). In contrast, gamma delta ( $\gamma\delta$ ) T cells are mildly reduced (2-4-fold) in DKO mice compared with WT controls (Figure 3.6C). This finding is in agreement with previous data showing E2A deficient mice have reduced numbers of  $\gamma\delta$  T cells (Bain et al., 1999b). Similarly,  $\gamma\delta$ T cell development is reduced 5-fold in Id1 transgenic mice (Kim et al., 1999). No changes in B cell, NK, or myeloid cell compartments were observed in DKO mice (data not shown). In agreement with these results, conditional disruption of E2A using the same *Lck*-driven Cre recombinase did not affect myeloid, NK, or B cell development

**Figure 3.5 Deletion of E2A and HEB results in a block at the DN stage of thymocyte development.** (A) Total thymocytes from  $HEB^{flox/flox} E2A^{flox/flox} Cre^{-}$ ,  $HEB^{flox/flox} E2A^{flox/flox} Cre^{+}$  (DKO), and  $LAT^{-/-}$  mice were harvested and subject to flow cytometric analysis after staining with CD8-FITC, CD5-PE, 7AAD, Mac-1-TC, Gr-1-TC, B220-TC, and CD4-APC. Following gating on forward and side scatter, 7AAD and Mac-1, Gr-1, and B220 staining was used to exclude dead cells and non-T cells from the analysis, respectively. Quadrant percentages are indicated.  $LAT^{-/-}$  thymocytes are shown as a control and are also blocked at the DN stage of development. (B) Relatively normal distribution of DN thymocyte subsets in DKO mice. Total thymocytes were stained with CD44-FITC, DX5-PE, CD4-TC, CD8-TC, B220-TC, Mac1-TC, Gr-1-TC, 7AAD, and CD25-APC to examine DN cell subsets. Following gating on forward and side scatter, cells negative for DX5, CD4, CD8, B220, Mac1, Gr-1, and 7AAD were used for analysis. Percentage of cells in each quadrant is indicated.  $LAT^{-/-}$  thymocytes are blocked at the DN3 stage of T cell development.





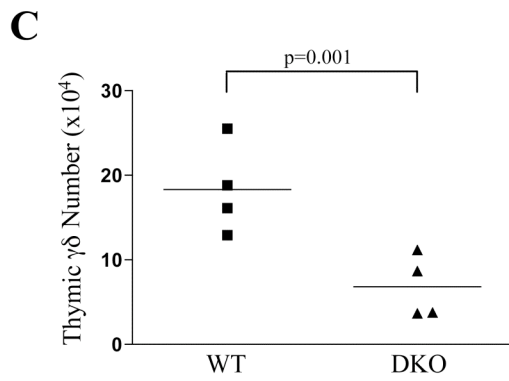
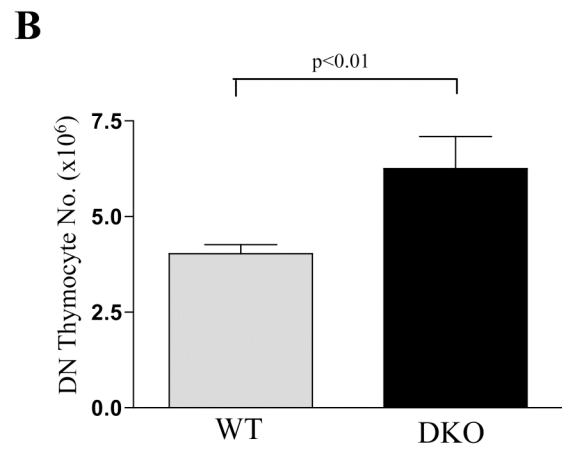
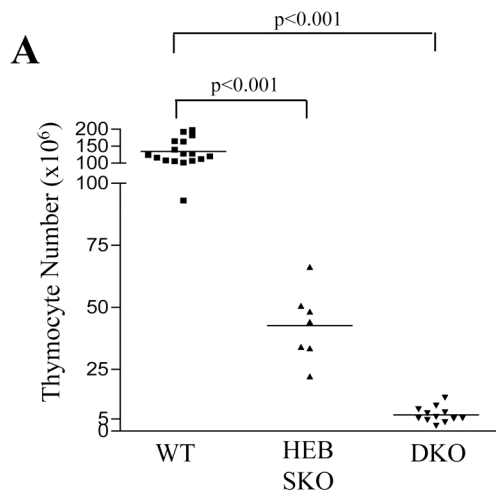
(Pan et al., 2002). Also, no changes in NK cell numbers were detected in Id1 transgenic mice (Kim et al., 1999). The increased phenotypic severity of the DKO in comparison to the HEB single knockout supports a cooperative role for E2A and HEB in thymocyte development.

### **3.2.4 TCR $\beta$ rearrangement is normal but pT $\alpha$ expression is dramatically reduced in DKO thymocytes**

Previous studies have indicated a requirement for E proteins in efficient TCR $\beta$  gene rearrangement (Barndt et al., 2000) and pT $\alpha$  gene transcription (Petersson et al., 2002; Tremblay et al., 2003). The successful completion of V(D)J  $\beta$ -recombination and subsequent pairing of the  $\beta$ -chain with pT $\alpha$  to form the pre-T cell receptor (pre-TCR) is absolutely required for the DN to DP stage transition during thymocyte development. We therefore investigated whether the defect in DKO thymocyte development could be due to the loss of proper TCR $\beta$ -rearrangement and/or pT $\alpha$  expression.

Touchdown PCR was used to evaluate V $\beta$ -D $\beta$ J $\beta$  recombination for both the V $\beta$ 5 and V $\beta$ 8 gene families, which together constitute approximately one quarter of the entire TCR $\beta$  repertoire (Wilson et al., 2001). LAT knockout and RAG2 knockout mice were included as positive and negative controls, respectively, to allow for direct comparison of V $\beta$ -D $\beta$ J $\beta$  rearrangement within the DN compartment. This assay revealed the presence of TCR $\beta$  V $\beta$ -D $\beta$ J $\beta$  recombination in DKO mice (Figure 3.7). Using the same assay, we were also unable to detect any differences in recombination between WT and DKO sorted DN3 and DN4 thymocytes (data not shown). Together, these results indicate the

**Figure 3.6 Thymic cellularity is perturbed in DKO thymocytes. (A)** Decreased total thymic cellularity in DKO mice. Total thymocytes from WT (n=17) and DKO (n=13) mice were harvested and counted using a Coulter Counter as per manufacturer recommendations. **(B)** Increased number of DN thymocytes in DKO. Numbers of DN cells in WT (n=17) and DKO mice (n=13) were calculated indirectly using the average DN % contribution to total thymocyte numbers by the formula: DN thymocyte number = total thymocyte number x DN %. Error bars represent standard deviation. **(C)** Decreased number of  $\gamma\delta$  T cells in DKO mice (n=4). Total thymocytes from WT and HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre<sup>+</sup> (DKO) mice were harvested, stained with CD4-FITC,  $\gamma\delta$ -PE, CD8-TC, CD4-TC, B220-TC, Mac-1-TC, Gr-1-TC, CD8-APC, and 7AAD and subject to flow cytometric analysis. After gating on forward and side scatter, cells negative for CD8, CD4, B220, Mac-1, Gr-1, and 7AAD were used for analysis. T-test P values between WT, SKO, and DKO cell numbers are indicated.



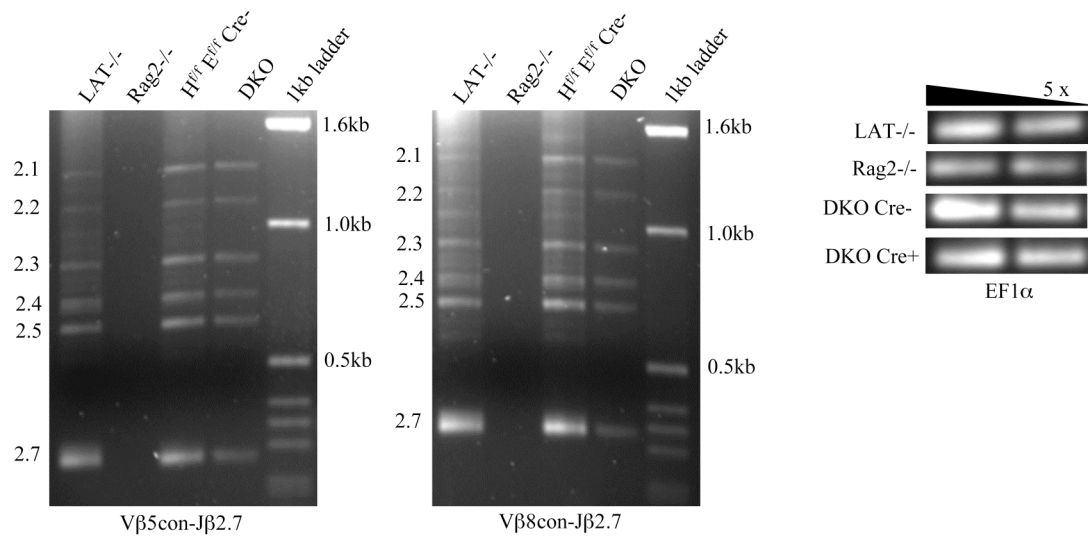
developmental block in DKO thymocyte development is unlikely due to defective TCR $\beta$  gene recombination.

We then evaluated pT $\alpha$  expression using real-time RT-PCR on sorted DN3 and DN4 thymocytes (Figure 3.8). Consistent with previous reports, pT $\alpha$  transcription is highly expressed in DN3 cells and down regulated in DN4 cells (Taghon et al., 2006). We detected a dramatic down-regulation of pT $\alpha$  expression in DKO thymocytes that was most evident in DN3 stage cells, the same developmental population in which thymocyte development is blocked in pT $\alpha$  knockout mice (Fehling et al., 1995). This result suggests that loss of pT $\alpha$  expression may partially contribute to the developmental defect of DKO mice. An E protein dose-related effect was also seen, as HEB single conditional knockouts displayed intermediate levels of pT $\alpha$  expression. These observations are consistent with reports that pT $\alpha$  is directly regulated by E proteins (Pettersson et al., 2002) and further confirms that E protein activity has been dramatically down regulated as early as the DN3 stage of development in DKO mice.

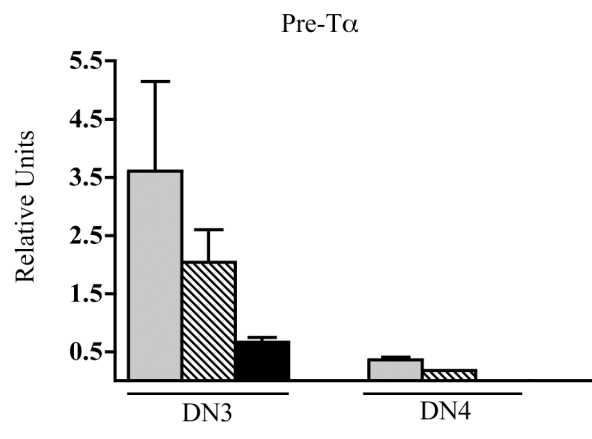
### **3.2.5 Loss of E proteins results in increased thymocyte apoptosis**

E proteins have been established as important regulators of cell survival during lymphocyte development. Interestingly, it appears that both disruption and overexpression of E proteins can induce apoptosis and only a narrow window of E protein activity is tolerated by the cell. E2A-deficient and E2A-HEB double deficient pre-B cells have been shown to undergo severe apoptosis (Lazorchak et al., 2006). Transgenic expression of Id1 leads to a dramatic reduction in thymocyte numbers and

**Figure 3.7 DKO thymocytes appear to have normal V to DJ TCR $\beta$  rearrangement.** Analysis of TCR $\beta$  recombination involving V $\beta$ 5 and V $\beta$ 8 gene families. Genomic DNA from total thymocytes was harvested and used for touchdown PCR analysis with the indicated V $\beta$ 5con-J $\beta$ 2.7 or V $\beta$ 8con-J $\beta$ 2.7 primers for the amplification of V-DJ recombination products. Rearrangements products involving J $\beta$ 2.1-7 (marked on the left of each gel) are detected in LAT $^{-/-}$ , HEB<sup>flox/flox</sup> E2A<sup>flox/flox</sup> Cre $^{-}$ , and DKO mice but not Rag2 $^{-/-}$  mice. A serial 5-fold dilution of EF1 $\alpha$  was used as a loading control.



**Figure 3.8 Pre-T $\alpha$  expression is regulated by E proteins in a dose-dependent fashion and is dramatically downregulated in DKO thymocytes.** Real time RT-PCR analysis of pT $\alpha$  expression. Messenger RNA was harvested from sorted WT, SKO, and DKO DN3 and DN4 thymocytes and cDNA was reverse transcribed and amplified by real time PCR. Shaded bars represent WT samples, hatched bars HEB SKO, and solid bars DKO samples. Relative units were calculated in reference to a standard curve generated by four 5-fold serial dilutions of WT thymic cDNA. Error bars represent the standard deviation of samples done in duplicate. Results are representative of three independent experiments.





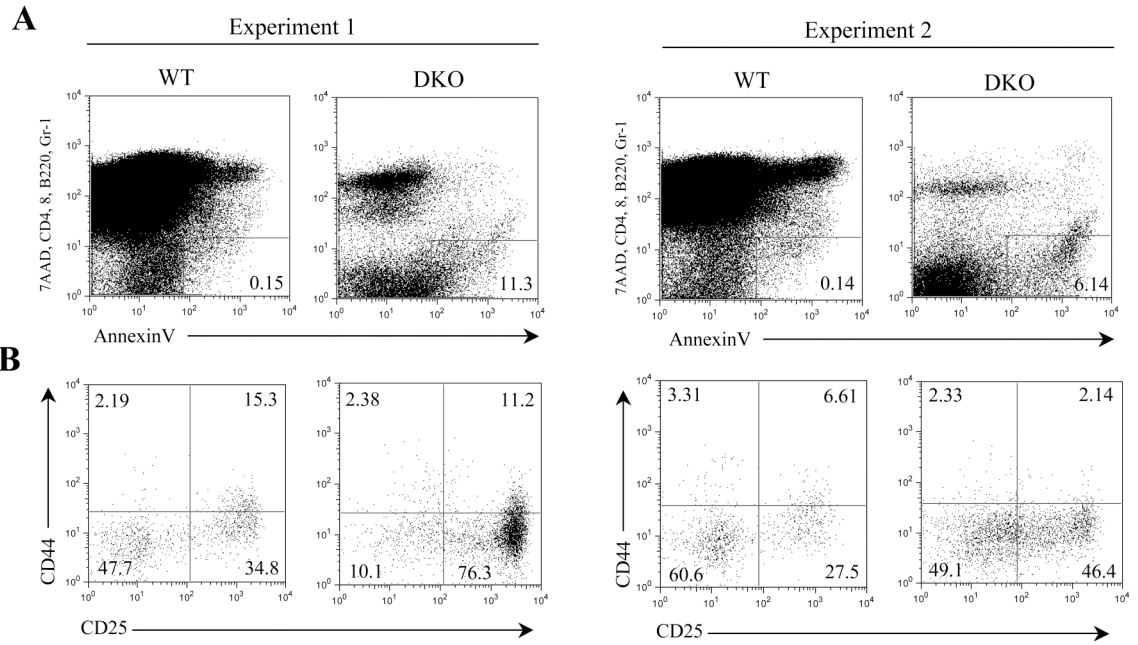
increase in apoptosis (Kim et al., 1999; Qi and Sun, 2004). Similarly, Id3 induces apoptosis in B lymphocyte progenitors (Kee, 2005). In addition, ectopic expression of E2A has been shown to induce cell death of E2A-deficient lymphomas (Engel and Murre, 1999). Therefore we examined whether the loss of HEB and E2A in our double conditional knockout perturbed cell survival as measured by AnnexinV staining.

In agreement with previous studies showing a role for E proteins in regulating cell survival, after accounting for a ~20-fold decrease in the total thymic cellularity of DKO mice, we observe a 2-4-fold increase in the total number of AnnexinV<sup>+</sup> DKO thymocytes (Figure 3.9). To determine which specific DN subset was responsible for the increase in apoptosis, we analyzed cells negative for CD8, CD4, B220, Gr-1, and 7AAD staining for CD44 and CD25 expression. Two experiments are shown to reflect the small degree of variability observed in DKO mice. Apoptosis of both DN1 and DN2 thymocytes from WT and DKO mice is similar. In contrast, after adjusting for the increased DN cellularity of DKO mice, in both Experiment 1 and Experiment 2 we detect approximately a 3-fold increase in DKO DN3 thymocyte apoptosis. DN4 thymocytes from DKO mice accordingly display less apoptosis than WT counterparts because there are fewer cells that have reached that developmental step.

### **3.3 Discussion**

We have described herein a HEB/E2A double-conditional knockout system and directly examined the T cell intrinsic contribution of E proteins to thymocyte development. Simultaneous disruption of HEB and E2A alleviates concerns of functional

**Figure 3.9. Disruption of E protein function results in increased thymocyte apoptosis.** (A) Total thymocytes from WT and DKO mice were harvested and stained with CD44-FITC, AnnexinV-PE, 7AAD, CD4-TC, CD8-TC, B220-TC, Gr-1-TC and CD25-APC. Cells negative for CD4, CD8, B220, Gr-1 and 7AAD were used for the analysis of AnnexinV expression. (B) AnnexinV positive cells from (A) were gated for analysis of CD44 and CD25 to discern among the different DN populations. Two independent experiments are shown.



compensation that have influenced the results of earlier studies on single E protein knockouts (Bain et al., 1997; Barndt et al., 1999). Also, by using a T cell-specific deletion system, we avoid interpretive complications caused by the broad lineage range of E protein expression. Using this model, we have identified a novel defect in thymocyte development that is expectedly more severe than that of E2A or HEB single knockouts. Although a third E protein (E2-2) exists in mammals, its involvement in thymocyte development is uncertain. While some authors have suggested that E2-2 plays a minor role in very early thymocyte development (Bergqvist et al., 2000), others have reported normal T cell development in E2-2<sup>-/-</sup> mice and that E2-2 is undetectable in the thymus (Zhuang et al., 1996). Although the possibility of E2-2 compensating for the loss of HEB and E2A formally exists, the severe block in thymocyte development in the DKO model suggests that E2-2 is unlikely to be a significant contributor to E protein function in early thymocytes or is unable to substitute for the concurrent loss of HEB and E2A. Importantly, it should be noted that it is unlikely we are missing any early HEB:E2A heterodimer-dependent function in thymocyte development due to the nature of our deletion construct. This claim is supported by the findings that neither HEB<sup>bm</sup> nor HEB knockouts display any DN1 or DN2 thymocyte defects. This suggests that in early T cell development, E2A homodimers may constitute the predominant functional dimer pair, and it is only after the DN2 stage that HEB:E2A heterodimers are important.

DKO mice exhibited a complete block in thymocyte development before the DN to DP transition, much like that of previously described HEB<sup>bm</sup> mice (Barndt et al., 2000). While both of these models theoretically abrogate E2A and HEB function, their

mechanism is different. The dominant-negative nature of the HEB<sup>bm</sup> construct allows for the potential inhibition of any bHLH complex it forms, not necessarily just E2A and HEB. Also, the HEB<sup>bm</sup> model is a genetic manipulation and is not T cell-specific. In contrast, the DKO model specifically disrupts HEB and E2A in T cells by using *Lck*-Cre to specifically drive Cre expression.

Two important phenotypic discrepancies were observed between HEB<sup>bm</sup> and DKO mice. First, DKO thymi contained a substantial number of DN4 cells, which were nearly absent in HEB<sup>bm</sup> mice. Second, recombination at the TCR $\beta$  locus was found to be normal in DKO thymocytes, whereas TCR $\beta$  V-DJ recombination was severely disrupted in HEB<sup>bm</sup> mice. Caution however, must be exercised in regards to interpreting the results of the TCR $\beta$  recombination status of DKO mice. While touchdown PCR is a sensitive assay capable of detecting TCR rearrangement, it is not a quantitative assay as performed and subtle defects in recombination may pass undetected. Both differences between the DKO and HEB<sup>bm</sup> model can most likely be attributed to incomplete Cre-mediated E2A and/or HEB deletion at the onset of V(D)J rearrangement. As our deletion analysis shows, there is still a small amount of floxed E protein alleles remaining in DKO thymocytes at later DN developmental stages. Also important to consider is that although deletion may have occurred on the gene level, protein products may persist. Finally, increased apoptosis in DKO thymocytes may eliminate a significant proportion of cells that have deleted E2A and HEB from our analysis. This would result in an underestimation of E protein deletion in DKO thymocytes. Regardless, the fact that virtually no cells progress past the DN stage indicates that deletion and protein levels are

extinguished by the DP stage of development. This is supported by the presence of only deleted alleles in DP thymocytes from E2A conditional knockout using the same *Lck* transgene to drive Cre expression (Pan et al., 2002).

Pre-T $\alpha$  expression has been shown to be regulated by E proteins (Herblot et al., 2000; Takeuchi et al., 2001; Tremblay et al., 2003). Accordingly, disruption of pT $\alpha$  expression in DKO thymocytes was expected assuming HEB and E2A deletion would be significant by the DN3 stage of development. We also observe a dose-dependent effect on pT $\alpha$  expression, as HEB single conditional knockouts display an intermediate level of expression between the WT and DKO. Functional E boxes are found in both the enhancer and promoter regions of pT $\alpha$ , although the latter is thought to be more important for E protein regulation. Interestingly, both regulatory elements have been found to bind HEB:E2A heterodimers in a stage-specific manner, suggesting a level of pT $\alpha$  (and therefore beta selection) regulation based on E protein function (Takeuchi et al., 2001; Tremblay et al., 2003). Disruption of pT $\alpha$  results in the elimination of pre-TCR signaling. While pT $\alpha$  knockout mice demonstrate a marked but incomplete block at the DN3-4 transition (Fehling et al., 1995), we only observe a moderate level of DN3 cell accumulation. This difference is again likely due to the incomplete loss of pT $\alpha$  expression in DKO thymocytes.

Importantly, in contrast to the developmental block induced by the disruption of pre-TCR signaling (Fehling et al., 1997; Fehling et al., 1995), the developmental block in HEB<sup>ko</sup> or HEB<sup>bm</sup> mice cannot be rescued by the introduction of a functional TCR transgene or  $\alpha$ CD3 $\epsilon$  treatment (Barndt et al., 1999; Barndt et al., 2000) (Y. Zhuang

unpublished results). This finding suggests the existence of currently unappreciated functions of E proteins in T cell development that are independent of TCR $\beta$  gene rearrangement and pre-TCR expression.

Engel et al. demonstrated that the DN3 block in T cell development normally associated with a defect in TCR recombination (and hence pre-TCR signaling) is avoided in E2A-deficient mice (Engel et al., 2001). This aberrant developmental circumvention suggests that E proteins are necessary to arrest inappropriate thymocyte maturation in the absence of successful pre-TCR signaling. The distinct phenotypes observed between this and our DKO model may be explained by a difference in the E protein dosage. In our system, both E2A and HEB are deleted, thus E protein activity is essentially abrogated. In contrast, in an E2A-deficient mouse such as the one employed by Engel et al., substantial E protein activity may persist. This suggests that a small amount of E protein activity is required for the DN to DP transition.

The observed increase in DKO thymocyte apoptosis is in agreement with other studies that reveal a role for E proteins in cell survival (Engel and Murre, 1999; Kee, 2005; Kim et al., 1999; Park et al., 1999). The finding that DN3 thymocytes from DKO mice are the primary subset responsible for the increase in apoptosis reflects the fact that pT $\alpha$  expression and pre-TCR signaling is required for survival and progression of thymocytes to the DN4 and DP stages of development (Fehling et al., 1995; Mombaerts et al., 1992a; Mombaerts et al., 1992b; Shinkai et al., 1992).

While the mechanism of apoptosis induction in DKO thymocytes is unclear, a number of possibilities may be proposed. E proteins may normally suppress apoptosis

through the activation of anti-apoptotic gene expression. However, this hypothesis is unlikely because transgenic *bcl-2* could not rescue apoptosis induced by ectopic expression of *Id3* (at least in B lymphocyte progenitors) (Kee, 2005). Another possibility is that a block in thymocyte development triggers apoptosis. In this case, the inability to progress may induce apoptosis by preventing cells from obtaining necessary survival signals such as pre-TCR signaling. Lastly, it is possible that the increased proliferation of DKO thymocytes triggers apoptosis. As previously mentioned, differentiation and proliferation need to be precisely coordinated during development. If cell cycle is inappropriately advanced during a critical developmental step, apoptosis may ensue. In the case of V(D)J recombination, the cell may interpret RAG-induced double-stranded DNA breaks as DNA damage and undergo apoptosis. This hypothesis is supported by Kim et al., who found TCR recombination events in apoptotic and dead *Id1* transgenic thymocytes (Kim et al., 1999).



**CHAPTER 4**  
**E PROTEINS REGULATE THYMOCYTE PROLIFERATION**

## 4.1 Introduction

In the last chapter we showed that simultaneous deletion of E2A and HEB in developing thymocytes leads to a severe developmental block at the DN to DP stage transition, a dramatic reduction of pT $\alpha$  expression, and increased apoptosis. In addition, we observed an increase in DKO DN thymic cellularity. These findings are in agreement with studies indicating a role for E proteins in the regulation of cellular differentiation, apoptosis, and proliferation. In this chapter we turn our focus to the effects of E protein deletion on proliferation. Although the influence of E proteins on differentiation is well-characterized, the proliferative aspect of E protein function is less understood.

The current model of E protein function is that E proteins inhibit proliferation and promote differentiation. Support for such a model comes from E2A knockout mice, which demonstrate both an early block in T cell development and an enhancement of T cell lymphoma development (Bain et al., 1997; Yan et al., 1997). Similar results from E protein disruption have been found in HEB<sup>ko</sup> and HEB<sup>bm</sup> mice (Barndt et al., 1999; Barndt et al., 2000 and Y. Zhuang unpublished results).

Studies on Id proteins, which antagonize E protein activity, have also contributed to our understanding of E proteins in regards to their effects on proliferation. Opposite to E proteins, high levels of Id expression have been shown to favor proliferation and inhibit differentiation (Benezra et al., 1990). In addition, while E2A has been shown to block the advancement of cells from the G<sub>1</sub>- to S-phase, Id proteins promote the G<sub>1</sub>- to S-phase transition (Barone et al., 1994; Benezra et al., 1990; Hara et al., 1994; Peverali et al.,

1994; Sun et al., 1991). In consideration of these roles for Id proteins, it is not surprising that overexpression of Id proteins results in lymphoma development (Kim et al., 1999).

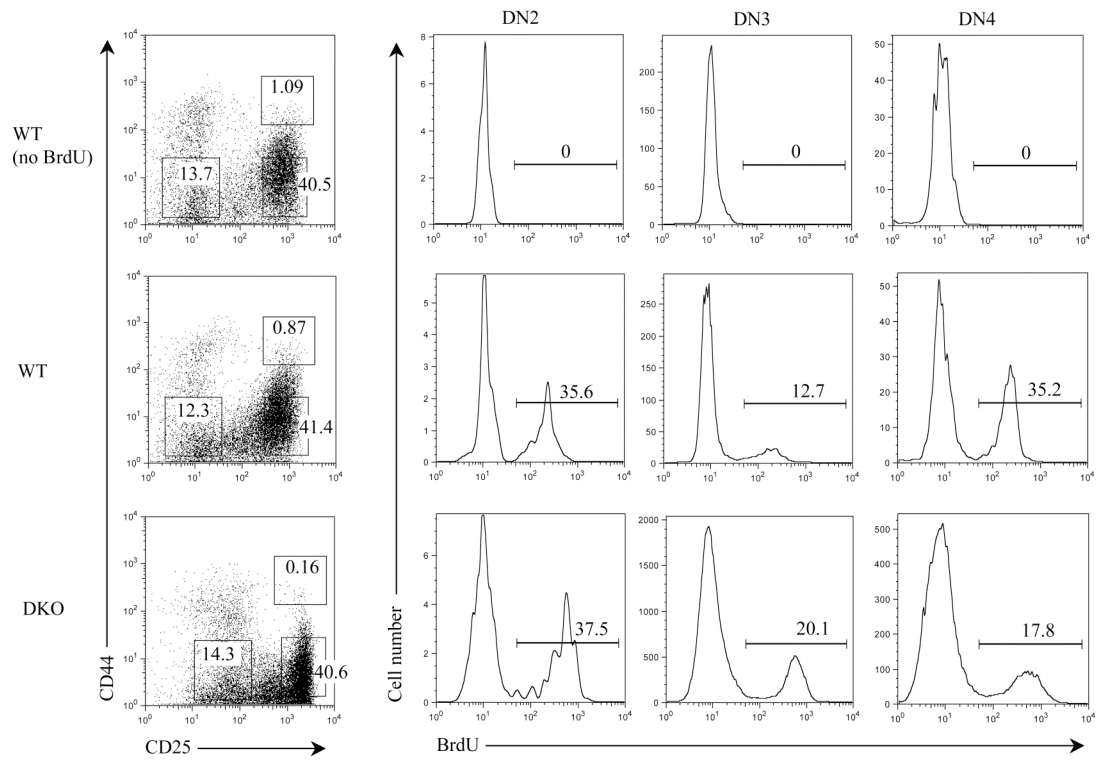
To more closely investigate the observed increase in DN thymocyte numbers in DKO mice and characterize the involvement of E2A and HEB in proliferation, we chose to directly examine proliferation of DKO thymocytes *in vivo* and *in vitro*. We find that these developmentally arrested thymocytes exhibit increased proliferation *in vivo* and dramatic expansion *ex vivo* in response to IL-7 signaling. These results suggest that E2A and HEB are not only critical for T cell differentiation but also necessary to retain developing thymocytes in cell cycle arrest prior to pre-TCR expression.

## **4.2 Results**

### **4.2.1 DKO thymocytes undergo enhanced proliferation *in vivo***

An increase in the total number of DN cells in DKO mice suggests the presence of enhanced cell proliferation resulting from the loss of E proteins. Accordingly, we chose to investigate *in vivo* thymocyte proliferation in DKO mice by measuring BrdU incorporation. In agreement with previous studies that indicate normal DN2 cells have a greater proliferative capacity than DN3 cells (Tourigny et al., 1997), WT DN2 thymocytes proliferate more robustly than DN3 cells (Figure 4.1). However, although DN2 thymocytes from both WT and DKO mice appear to undergo a similar degree of proliferation, DKO DN3 thymocytes proliferate more than 1.5-fold the amount of their

**Figure 4.1 Thymocytes from DKO mice hyper-proliferate *in vivo*.** Proliferation analysis of WT and DKO thymocytes. Mice were injected with 1mg BrdU and sacrificed 4 hours later. Total thymocytes were stained with BrdU-FITC, CD44-PE, CD4-TC, CD8-TC, B220-TC, Mac-1-TC, Gr-1-TC and CD25-APC. Following gating on forward and side scatter, cells staining negative for CD4, CD8, B220, Mac-1 and Gr-1 were analyzed for CD44/25 expression. The boxed regions on the CD44/25 plot corresponding to DN2, DN3, and DN4 cells were analyzed for BrdU incorporation. Cell percentages in the boxed regions are indicated. Histograms indicating the percentage of BrdU positive cells for each DN subset examined are shown. Results are representative of two experiments.

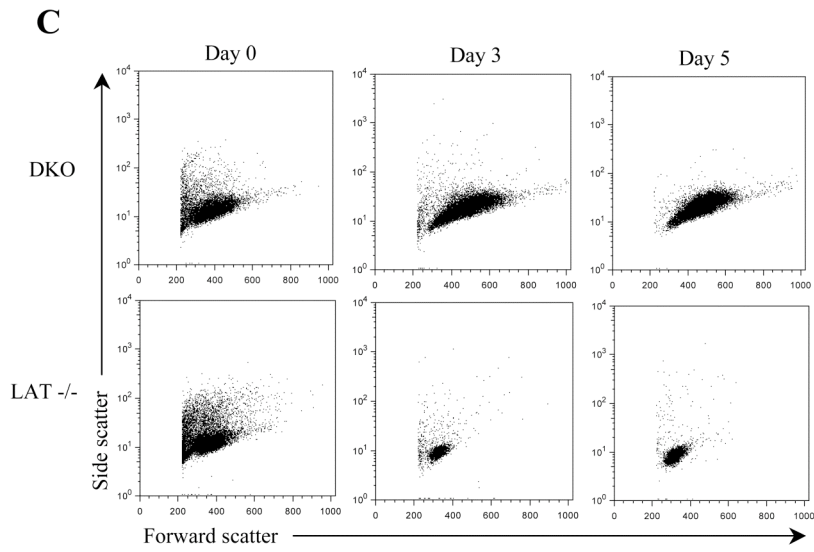
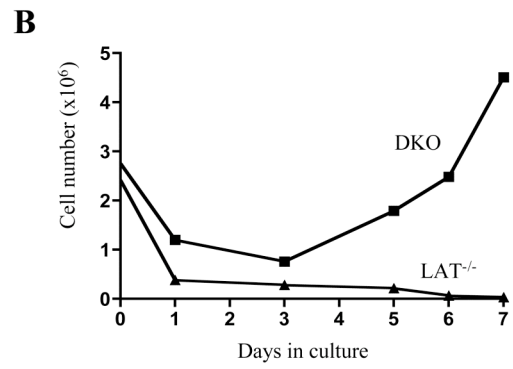
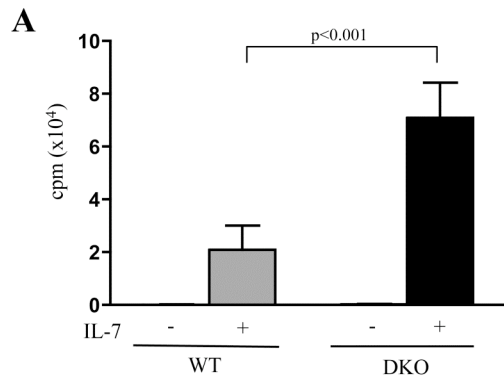


WT counterparts (12.7% vs. 20.1%, respectively). This result suggests that the loss of E proteins during T cell development releases DN3 thymocytes from the G<sub>1</sub> phase retention and allows for cell cycle progression. In contrast, DN4 cells from DKO mice display a reduction in proliferation compared with WT cells (Figure 4.1). Because proliferation of DN4 cells is strictly dependent on pre-TCR signaling, the reduction of BrdU incorporation in DN4 DKO cells can likely be attributed to the severely compromised pT $\alpha$  expression (and therefore pre-TCR signaling) observed in DKO thymocytes.

#### **4.2.2 IL-7 dependent proliferation of DKO thymocytes *ex vivo***

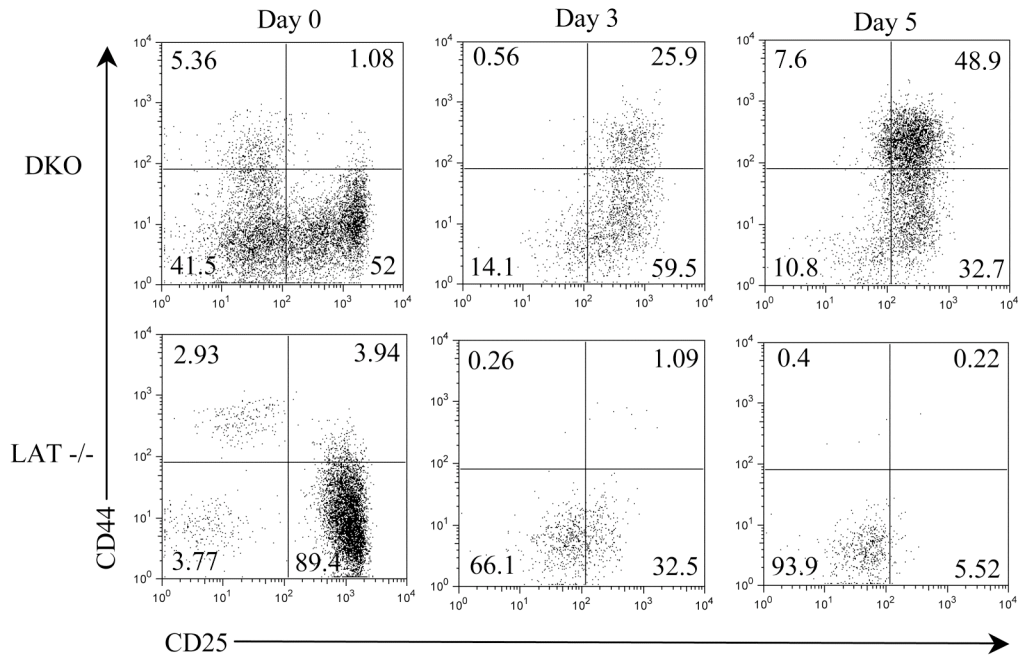
IL-7 is an essential cytokine for normal DN thymocyte expansion and survival (reviewed by Jiang et al., 2005). The increased proliferation of DKO DN3 cells *in vivo* prompted us to investigate the responsiveness of DKO thymocytes to IL-7 in an *ex vivo* culture system. Total DN cells were isolated from WT or DKO mice and placed in culture in the presence or absence of IL-7 and pulsed with [<sup>3</sup>H]-thymidine. Although both WT and DKO DN cells exhibit IL-7-dependent proliferation, after 48h in culture DKO thymocytes display approximately 3-4-fold higher [<sup>3</sup>H]-thymidine incorporation than WT cells (Figure 4.2A). Next we investigated the kinetics of proliferation by measuring cell numbers periodically throughout the culture period. In these experiments we utilized LAT<sup>-/-</sup> DN thymocytes, which respond similarly to WT DN cells in our culture system (data not shown). In contrast to LAT<sup>-/-</sup> DN cells that fail to proliferate, we observed a significant increase in the number of DKO thymocytes following 7 days of culture in the presence of IL-7 (Figure 4.2B). This expansion follows a transient

**Figure 4.2 DKO thymocytes are hyper-proliferative *ex vivo* in response to IL-7.** (A) DKO thymocytes undergo enhanced proliferation in the presence of IL-7.  $1 \times 10^5$  purified DN thymocytes from WT or DKO mice were cultured in the presence or absence of 10ng/mL IL-7 for 48h and pulsed with [ $^3$ H]-thymidine 6h before harvesting and determining radioactive incorporation. Error bars represent the standard deviation of samples done in triplicate. Results are representative of three independent experiments. P values between WT and DKO samples are indicated. (B) Increase in cell number of DKO but not LAT<sup>-/-</sup> DN thymocytes following a 7-day culture in the presence of 10ng/mL IL-7. Plot shown is representative of three independent experiments. (C) DKO but not LAT<sup>-/-</sup> thymocytes display a blasting phenotype following *ex vivo* culture in the presence of 10ng/mL IL-7. Total thymocytes from DKO and LAT<sup>-/-</sup> culture were harvested at day 0 (pre-culture), day 3 and day 5 and analyzed by flow cytometry. Cells were stained with CD44-FITC, c-kit-PE, CD4-TC, CD8-TC, Mac-1-TC, B220-TC, 7AAD, and CD25-APC. Cells negative for CD4, 8, Mac-1, B220, and 7AAD were gated and used for forward and side scatter analysis.





**Figure 4.3 The emergence of DN2-like phenotype cells in DKO but not LAT<sup>-/-</sup> thymocytes IL-7 culture.** Total thymocytes from DKO and LAT<sup>-/-</sup> culture were harvested at day 0 (pre-culture), day 3 and day 5 and analyzed by flow cytometry for CD44 and CD25 expression following gating on forward and side scatter and exclusion of CD4, 8, Mac-1, Gr-1, B220, and 7AAD signals. Quadrant percentages are indicated.



decrease in DKO cell numbers, suggesting that cell death occurs in the majority of DN cells and only a subset is responsible for the increased proliferation. Consistent with an increase in cell number, the proliferating DKO cells show a blasting phenotype whereas the non-proliferating  $LAT^{-/-}$  cells show a decrease in cell size by both FACS (Figure 4.2C), and microscopic analysis (data not shown).

Interestingly, over the course of culture we observe the gradual emergence of DN2-like cells in DKO but not  $LAT^{-/-}$  cultures (Figure 4.3). These cells appear to be T lineage cells as evidenced by positive Thy1 staining and were confirmed to have a DN2 phenotype by expressing CD44, CD25, and CD117 (ckit) but not non-DN2 markers including CD4, CD8, CD5, DX5,  $\gamma\delta$  TCR, Gr-1, and Mac1 (data not shown). Also, there was no change in either  $DX5^{+}$  NK cells or  $\gamma\delta$  T cells during the culture period (data not shown). PCR analysis was used to quantify the deletion of E2A and HEB in the cultured cells. We show a significant, yet incomplete deletion of E2A and HEB in cultured DKO cells (Figure 4.4).

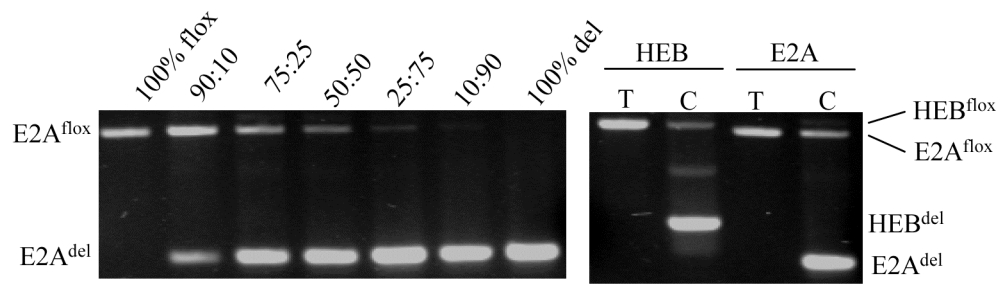
Because DKO DN3 cells hyper-proliferate *in vivo* yet we detect the expansion of a DN2-like population following *ex vivo* culture, we sought to determine which specific subset of DN thymocytes was responsible for the observed increase in proliferation. To address this question, DN2 and DN3 thymocytes from DKO mice were sorted and placed in culture independently in the presence or absence of IL-7. Cells were pulsed with [ $^3$ H]-thymidine on day 6 of culture and harvested at day 7. In agreement with results obtained from *in vivo* BrdU labeling experiments, DN3 cells from DKO mice hyper-proliferated in response to IL-7 signaling (Figure 4.5A).

Specifically, DN3 cell proliferation was approximately 10-fold that of DN2 cells from DKO mice. This increase in proliferation is accompanied by a DN2-like phenotypic shift over the course of the culture resembling that previously seen in total DN cell culture (Figure 4.5B). In addition, examination of the TCR $\beta$  V(D)J rearrangement status of Day 7 sorted DKO cells under these culture conditions revealed the presence of V-DJ recombination (Figure 4.5C). Together these results imply that DN3 cells are the primary cell subset responsible for the increased proliferation of DKO thymocytes in IL-7 culture. Also, these observations indicate a novel role for E proteins in antagonizing IL-7-dependent proliferation during early thymocyte development.

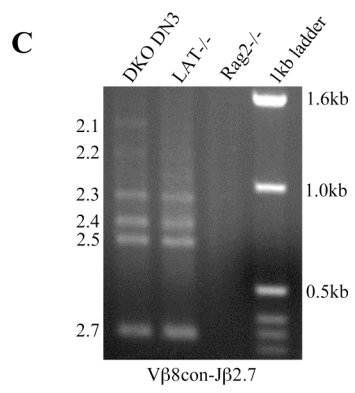
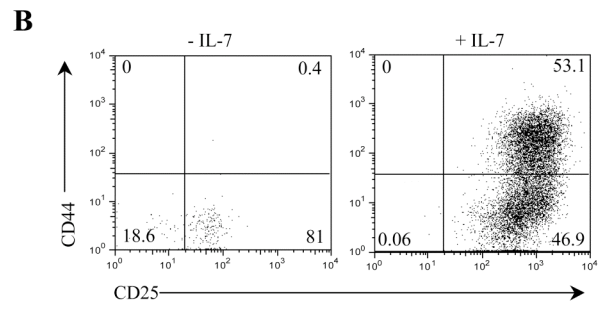
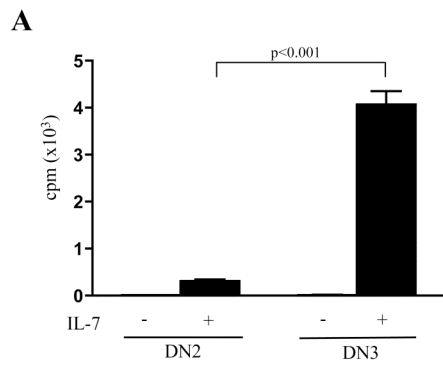
#### **4.2.3 Deregulation of cell cycle regulatory genes**

We next chose to investigate the mechanism(s) responsible for the increased proliferation in DKO thymocytes. Progression through the cell cycle is tightly regulated by the balance of cyclins and cyclin-dependent kinase inhibitors (CDKIs) (Kozar et al., 2004; Sherr and Roberts, 1999). E proteins have been shown to be important in the regulation of cell cycle, possibly by influencing CDKIs and cyclin expression. E proteins have been implicated as being positive transcriptional regulators of p15<sup>Ink4b</sup>, p16<sup>Ink4a</sup>, and p21<sup>Cip1</sup> (Herblot et al., 2002; Pagliuca et al., 2000; Prabhu et al., 1997). In addition, E proteins have been shown to activate expression of cyclins D2 and D3 (Zhao et al., 2001). Therefore, we evaluated the effect of E protein deletion on the expression of these cell cycle regulators using real-time RT-PCR on sorted DN3 and DN4 thymocytes and *ex vivo* cultured cells (Figures 4.6A and 4.6B).

**Figure 4.4 HEB and E2A deletion analysis of cultured cells.** An E2A serial dilution PCR with known ratios of floxed vs. deleted alleles is shown for reference to estimate the efficiency of deletion (analogous to assay used in Fig 1C for HEB deletion). Toe (T) DNA from HEB<sup>flox/flox</sup>E2A<sup>flox/flox</sup> Cre<sup>-</sup> mice and DNA from Day 5 cultured (C) DKO cells were assayed for HEB or E2A deletion. Floxed and deleted alleles for HEB and E2A are indicated.



**Figure 4.5 DN3 thymocytes are responsible for the increased proliferation of DKO cells in IL-7 culture.** (A) DN3 cells undergo enhanced proliferation in the presence of IL-7. Thymocytes from DKO mice were sorted into DN2 and DN3 populations based on CD44, CD25, and c-kit staining. DN2 cells were identified as CD44<sup>+</sup>CD25<sup>+</sup>c-kit<sup>+</sup> while DN3 cells were CD44<sup>-</sup>CD25<sup>+</sup>c-kit<sup>-</sup>. 1x10<sup>4</sup> cells were cultured in the presence or absence of 10ng/mL IL-7 for 7 days and pulsed with [<sup>3</sup>H]-thymidine 14 hrs before harvesting and determining radioactive incorporation. Error bars represent the standard deviation of 3-5 samples. Results are representative of two independent experiments. T-test P values between DKO DN2 and DN3 samples are indicated. (B) DKO DN3 thymocytes undergo a phenotypic shift to DN2-like cells following 7 days of culture in the presence of 10ng/mL IL-7. Cells were stained with CD44-FITC, c-kit-PE, CD4-TC, CD8-TC, Mac-1-TC, B220-TC, 7AAD, and CD25-APC. Following gating on forward and side scatter, cells negative for CD4, 8, Mac-1, B220, and 7AAD were used in the analysis. Quadrant percentages are indicated. (C) Cultured DN3 cells show normal V(D)J recombination. Genomic DNA from D7 cultured thymocytes was harvested and used for touchdown PCR analysis with the indicated primers for the amplification of V<sub>β</sub>8-J<sub>β</sub>2.1-7 recombination products (marked on the left of each gel) are as indicated.





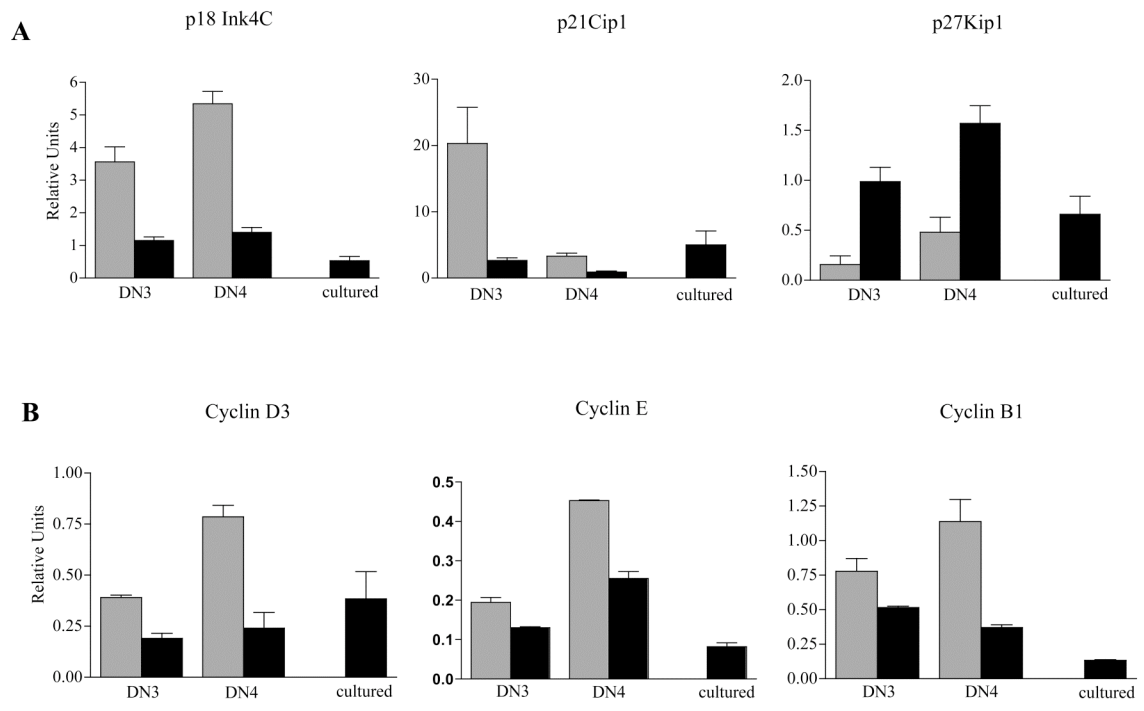
In WT mice, p21<sup>Cip1</sup> is highly expressed at the DN3 stage and downregulated at the DN4, whereas both p18<sup>Ink4c</sup> and p27<sup>Kip1</sup> are slightly increased from the DN3 to DN4 stage of development. Analysis of DKO thymocytes showed a dramatic down-regulation of p18<sup>Ink4c</sup> and p21<sup>Cip1</sup> expression in both the DN3 and DN4 stages, suggesting a positive role for E proteins in regulating these two CDKI genes. In contrast, p27<sup>Kip1</sup> is upregulated in DKO mice, indicating that it is negatively regulated by E proteins.

Expression of cyclin D3, cyclin E, and cyclin B1 in WT samples increased as cells transitioned from the DN3 to DN4 stage of thymocyte development. DKO thymocytes consistently exhibited decreased expression of all cyclins analyzed, particularly in DN4 cells. Analysis of DKO thymocytes cultured *ex vivo* in the presence of 10ng/mL IL-7 revealed that CDKI and cyclin expression was not altered by the culture conditions, as expression was similar to DKO DN3 and DN4 thymocytes (Figures 4.6A and 4.6B). Together, these results imply that E proteins function in a complex manner to influence the expression of cell cycle regulatory genes and cell cycle progression.

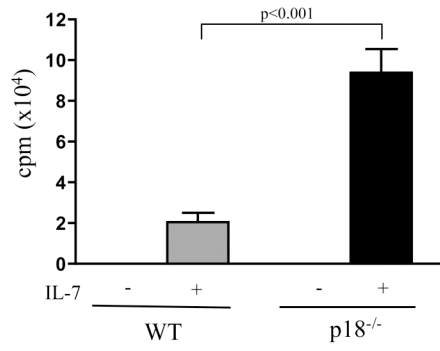
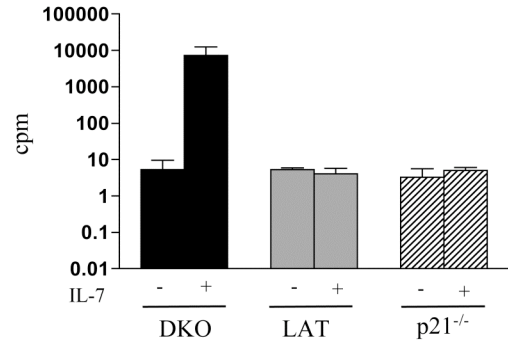
#### **4.2.4 p18<sup>Ink4c</sup>-deficient thymocytes hyper-proliferate *ex vivo***

Disruption of CDKI expression has been linked to aberrant proliferation. Because our real time RT-PCR data indicates that p18<sup>Ink4c</sup> and p21<sup>Cip1</sup> expression is down regulated in both DN3 and DN4 DKO thymocytes, we sought to determine whether the elimination of p18<sup>Ink4c</sup> or p21<sup>Cip1</sup> alone is sufficient to cause cell cycle deregulation in DN cells. Using a proliferation assay of cultured DN thymocytes from p18<sup>Ink4c</sup> or p21<sup>Cip1</sup> knockout mice, we show that loss of p18<sup>Ink4c</sup> results in approximately a 4-5 fold increase

**Figure 4.6 Thymocytes from DKO mice display altered levels of CDKI and cyclin expression.** Real time RT-PCR analysis of cyclin-dependent kinase inhibitors (CDKIs) p18<sup>Ink4c</sup>, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> as well as cyclins D1, E, and B1 in sorted DN3 and DN4 thymocytes. Shaded and solid bars represent HEB<sup>flox/flox</sup>E2A<sup>flox/flox</sup> Cre<sup>-</sup> and DKO samples, respectively. Error bars represent the standard deviation of samples done in duplicate. Results are representative of 2-3 independent experiments with independently sorted thymocytes. Experiments with cultured cells were done with one batch of cDNA and performed in duplicate. Relative units were calculated in reference to a standard curve generated by four 5-fold serial dilutions of WT thymic cDNA.



**Figure 4.7 p18-deficient but not p21-deficient thymocytes hyper-proliferate *ex vivo* in the presence of IL-7.** (A) p18<sup>-/-</sup> thymocytes and (B) p21<sup>-/-</sup> thymocytes. For both (A) and (B) 1x10<sup>5</sup> purified DN thymocytes from WT and p18 or p21 knockout mice were cultured in the presence or absence of 10ng/mL IL-7 for 48h and pulsed with [<sup>3</sup>H]-thymidine 6h before harvesting and determining radioactive incorporation. Results are representative of three independent experiments. T test P values between WT and p18<sup>-/-</sup> samples are indicated. Error bars represent the standard deviation of samples done in triplicate.

**A****B**

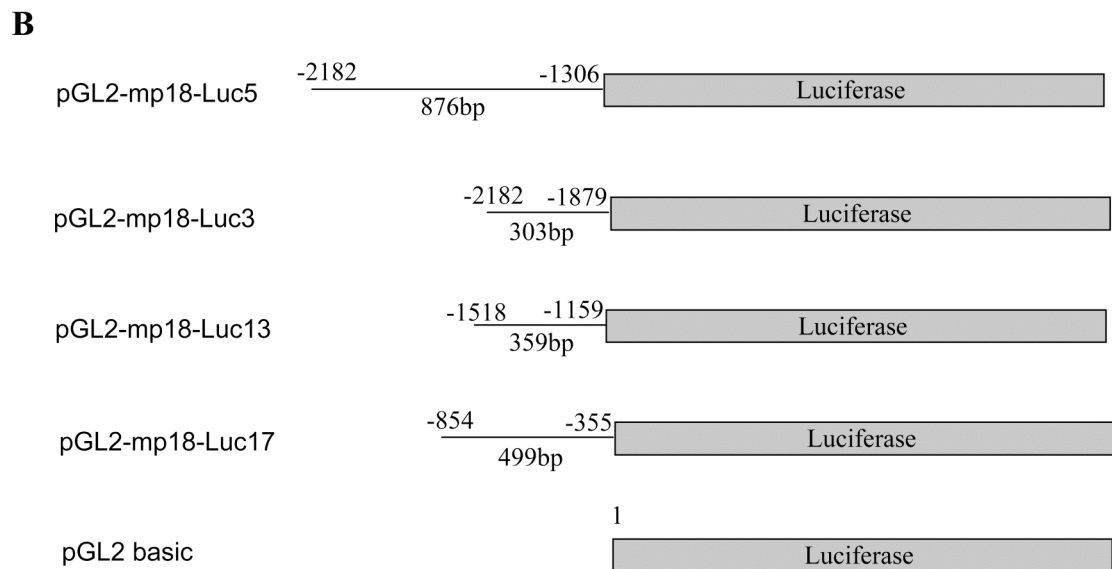
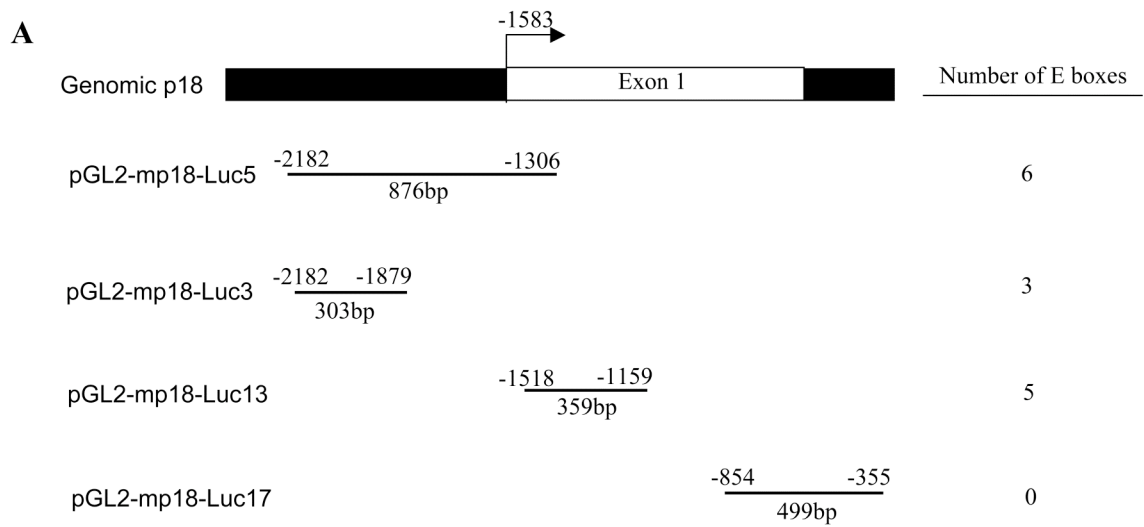
in proliferation (Figure 4.7A), suggesting that p18<sup>Ink4c</sup> is indeed an important CDKI for the suppression of proliferation in DN thymocytes. In contrast, no change in proliferation was detected for p21<sup>Cip1</sup> (Figure 4.7B).

#### **4.2.5 E proteins appear to regulate p18<sup>Ink4c</sup> expression in an indirect manner**

To determine whether p18<sup>Ink4c</sup> expression is directly regulated by E proteins, we performed dual luciferase reporter assays. For this purpose, we obtained a panel of vectors containing serially truncated p18<sup>Ink4c</sup> 5' promoter regions used to drive luciferase expression (Figure 4.8) (a generous gift from Yue Xiong; UNC Chapel Hill). These deletion constructs contain potential E box binding sites and therefore are valuable in determining E protein regulation of p18<sup>Ink4c</sup> transcription. Jurkat, EL4 (data not shown), or p5424 thymoma cells were transfected with the p18<sup>Ink4c</sup> 5' promoter constructs with or without an E47 expression vector. In all cell lines examined, no direct regulation of p18<sup>Ink4c</sup> by E47 was detected as measured by normalized luciferase expression, suggesting that E proteins may influence p18<sup>Ink4c</sup> indirectly or through E box sites not included in the constructs (Figure 4.9A and B).

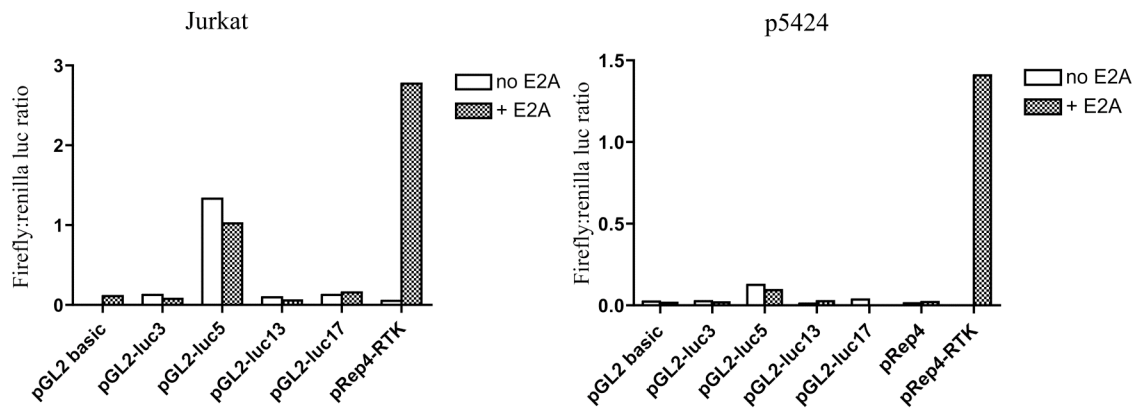
As another possibility, the transcription factor menin encoded by the *Men1* (multiple endocrine neoplasia) gene has been shown to positively regulate p18<sup>Ink4c</sup> expression (Bai et al., 2007). We reasoned that if E proteins activate *Men1* expression, disruption of E protein function could result in the downregulation of *Men1* and subsequently p18<sup>Ink4c</sup> expression. Real time PCR analysis of *Men1* expression in WT and DKO sorted DN3 and DN4 thymocytes revealed a minor role for E proteins in regulating

**Figure 4.8 Schematic diagram of p18<sup>Ink4c</sup> luciferase vectors.** (A) Orientation and location of promoter fragments in relation to genomic p18<sup>Ink4c</sup> sequence. The arrow indicates the transcription start site from the 5' upstream promoter. The number of E box sites contained within each promoter fragment is included. The length of each fragment and the corresponding nucleotide position is indicated, with the A nucleotide of the ATG codon being arbitrarily set to 1. Adapted from Phelps et al., 1998. (B) Promoter fragments were cloned into the pGL2-Basic luciferase reporter plasmid and used to drive luciferase expression following transfection with or without E2A into target cells.





**Figure 4.9 p18<sup>Ink4c</sup> reporter constructs are not transactivated by E2A overexpression.** Jurkat or p5424 cells were transiently transfected with various E box-containing p18 promoter constructs with or without an E47 expression vector and a renilla luciferase plasmid. pRep4-RTK is used as a positive control for E2A-dependent transcription. Cells were analyzed for luciferase expression 24h post-transfection. Results are representative of 4-5 independent experiments.

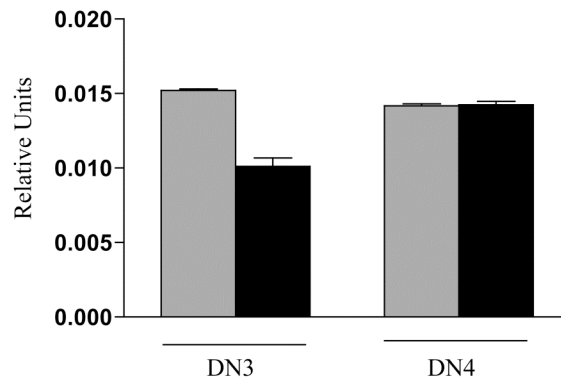


*Men1* expression at the DN3 but not the DN4 stage. *Men1* expression in DKO DN3 thymocytes was decreased approximately 30% compared with WT DN3 cells (Figure 4.10). This relatively minor decrease is unlikely to be the sole contributor of E protein-mediated regulation of p18<sup>Ink4c</sup> expression. Together these results suggest that additional unidentified mechanisms of E protein-regulated p18<sup>Ink4c</sup> transcriptional activation exist.

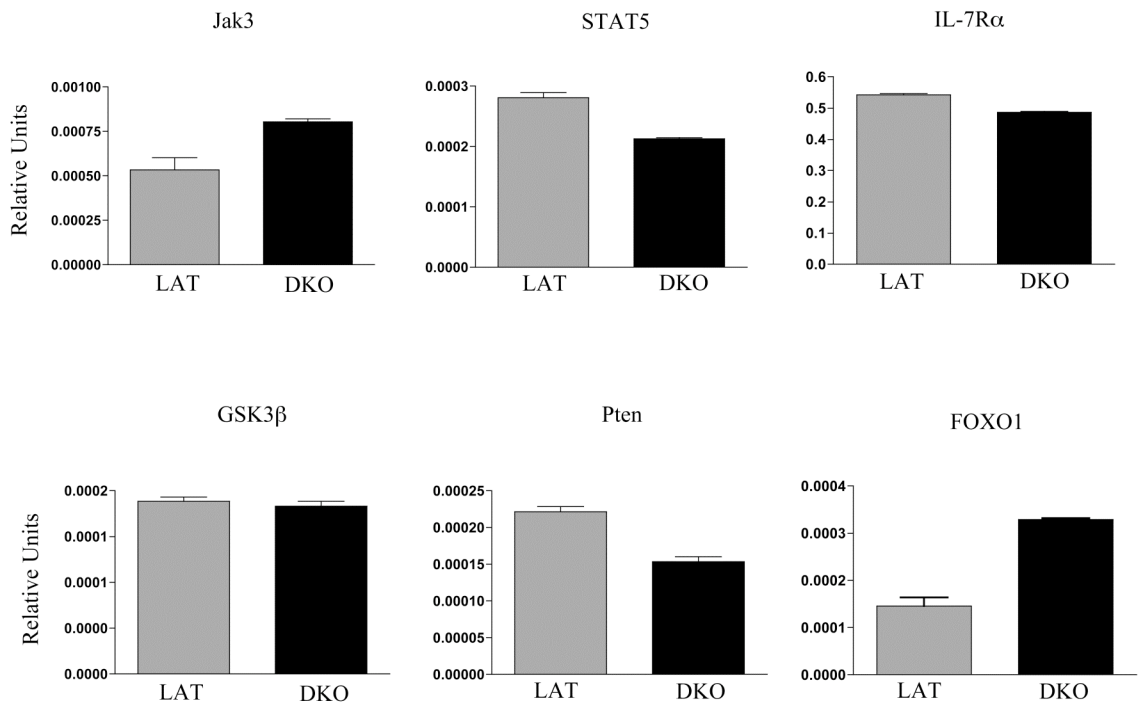
#### **4.2.6 Examination of IL-7 related signaling components**

IL-7 signaling is mediated by a number of signaling pathways including the Jak/STAT and MAPK pathways, and PI3K (reviewed by Jiang et al., 2005). To examine whether the disruption of E proteins alters the expression of IL-7 signaling molecules we examined a panel of targets by real time PCR (Figure 4.11). Jak3 and FOXO1 expression was slightly increased in DKO thymocytes in comparison to LAT cells. FOXO1 is a transcription factor involved in the anti-apoptotic effects of IL-7 signaling (Barata et al., 2004). Conversely, STAT5 and Pten were mildly reduced in DKO thymocytes. Although not directly induced by IL-7 signaling, Pten (phosphatase and tensin homolog) is a phosphatase that antagonizes PI3K function by removing the 3-phosphate of all PI3K-catalyzed products (Koyasu, 2003). GSK3 $\beta$  (glycogen synthase kinase) and IL-7R $\alpha$  expression was similar between DKO and LAT samples. Inactivation of GSK3 $\beta$  prevents cyclin D1 degradation, and thus acts to promote cell cycle progression (Diehl et al., 1998). Together these results suggest a minor role for E proteins in regulating the expression of IL-7 signaling molecules. Whether such mild changes in gene expression has any functional or phenotypic consequences remains to be determined.

**Figure 4.10 Disruption of E proteins results in a mild decrease in Men1 expression in DN3 thymocytes.** Real time RT-PCR analysis of Men1 expression in DN3 and DN4 thymocytes. Shaded and solid bars represent WT and DKO samples, respectively. Error bars represent the standard deviation of samples done in duplicate on a single batch of sorted cDNA. Results representative of two independent experiments. Relative units were calculated in reference to a standard curve generated by four 5-fold serial dilutions of WT thymic cDNA.



**Figure 4.11 E proteins do not appear to significantly influence the expression of molecules involved in IL-7 signaling.** Messenger RNA was harvested from total thymocytes from LAT (shaded bars) and DKO (solid bars) mice. cDNA was reverse transcribed and amplified by real time PCR. Error bars represent the standard deviation of samples done in duplicate. Results are representative of 2-3 independent experiments from the same batch of cDNA. Relative units were calculated in reference to a standard curve generated by four 5-fold serial dilutions of WT thymic cDNA.



#### **4.2.7 Notch signaling inhibits *ex vivo* phenotypic dedifferentiation of DKO thymocytes**

The intriguing finding of reverse differentiation of DKO thymocytes from the DN3 to DN2 stage in our *ex vivo* culture prompted us to further investigate this phenomenon. Because we do not see this phenotypic reversion *in vivo*, we sought to reconcile these differences by more-closely recapitulating the thymic environment *ex vivo* and determine the propensity of E protein-deficient thymocytes to undergo reverse differentiation. To accomplish this, we employed the use of OP9-DL1 system and analyzed DKO thymocytes in the context of a stromal cell layer that can support T cell differentiation to the mature T cell stage (Schmitt and Zuniga-Pflucker, 2006). In this *in vitro* culture system, activation of the Notch pathway by Delta-like 1 ligand allows for T cell development (Balciunaite et al., 2005; Schmitt and Zuniga-Pflucker, 2002). Total DKO thymocytes were cultured in the presence of 10ng/mL IL-7 with no stromal layer, OP9 cells (which lack Delta-like 1 expression), or OP9-DL1 cells. Over the course of 7 days in culture, we observe the characteristic reverse differentiation of DKO cells in the absence of any stromal layer. Similarly, DKO thymocytes cultured in the presence of OP9 stromal cells also underwent a DN3 to DN2 phenotypic conversion. In contrast, when DKO thymocytes were placed in culture with OP9-DL1 cells, a DN3 phenotype was maintained by the DKO cells (Figure 4.12). This novel finding suggests that Notch signaling can override the tendency for E protein-deficient thymocytes to undergo backwards differentiation and may enforce the forward differentiation of T cells. The reverse differentiation of DKO thymocytes cultured alone or in the presence of OP9 cells is dependent on IL-7 signaling, as cells cultured in the absence of IL-7 do not undergo a

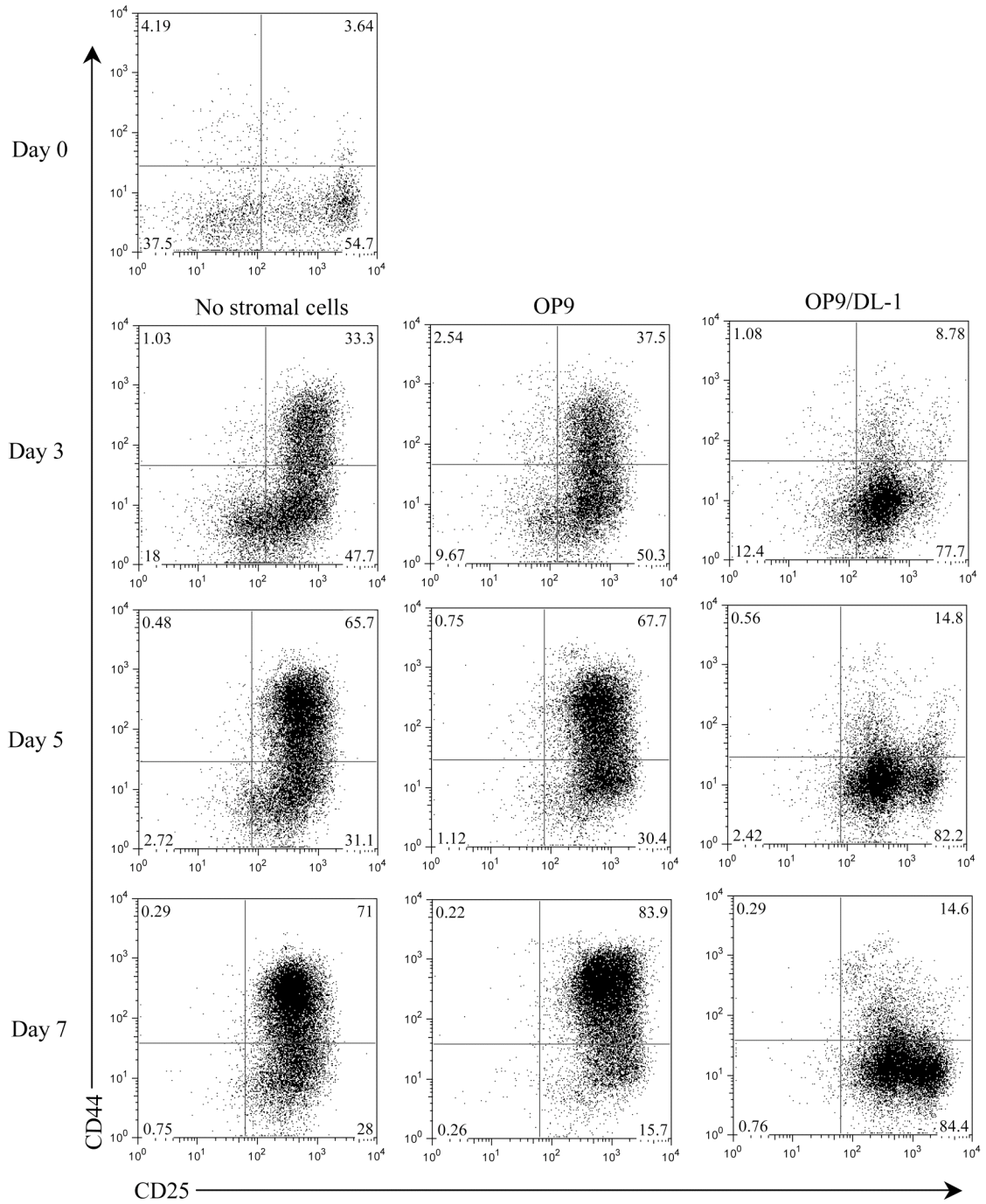


DN3 to DN2 phenotypic shift (data not shown). Similar degrees of proliferation were observed among the three culture conditions over the course of the experiment as measured by forward/side scatter analysis (data not shown). Also of note is that while Notch signaling is typically able to drive mature T cell differentiation, DKO thymocytes remain in the DN compartment and do not progress to more advanced stages of development (data not shown). This suggests Notch signaling cannot override the defect in thymocyte development caused by the disruption of E protein function.

### **4.3 Discussion**

Proliferation and differentiation are coordinately regulated during thymocyte development and are thought to be mutually exclusive. During the DN stages of development, cells must balance cell cycle progression with ongoing rearrangement of the TCR $\beta$  chain. Once proliferating DN2 thymocytes advance to the DN3 stage and commence TCR $\beta$  rearrangement, cell cycle is slowed or arrested. Following successful rearrangement and pre-TCR signaling, cells undergo another burst of proliferation (Hoffman et al., 1996; Laurent et al., 2004). Our study reveals a crucial role for E proteins to retain developing T cells in a non-proliferative state prior to pre-TCR expression. Specifically, *in vivo* BrdU labeling assays demonstrated an increased proliferation of DN3 thymocytes in DKO mice. This increased proliferative capacity was further supported by the *ex vivo* culture assay, in which DN3 thymocytes from DKO mice were found to expand in the presence of IL-7 more vigorously than controls.

**Figure 4.12 Notch signaling inhibits the DN2 phenotypic reversion of DKO thymocytes cultured ex vivo in the presence of IL-7.**  $1 \times 10^6$  total thymocytes from DKO mice were placed in culture alone or cocultured with OP9 or OP9-DL1 stromal cells in the presence of 10ng/mL IL-7. Cells were harvested at indicated time points and stained with CD44-FITC, ckit-PE, 7AAD, VCAM1-bio/strep-TC, CD4-TC, CD8-TC, B220-TC, Gr-1-TC, and CD25-APC. Cells negative for 7AAD, VCAM1, CD4, CD8, B220, and Gr-1 were analyzed for CD44 and CD25 expression. VCAM staining was used to reduce stromal cell contamination. Results are representative of two independent experiments.



Curiously, most proliferating cells in our *ex vivo* culture assay acquire canonical DN2 markers including CD44, CD25, and CD117 (c-kit) but not other lineage markers including Mac1,  $\gamma\delta$  TCR, or DX5 (to identify myeloid cells,  $\gamma\delta$  T cells, and NK cells, respectively). This phenotypic reversion suggests a novel and pivotal role for E proteins in promoting normal maturational progression while simultaneously inhibiting the backwards differentiation of DN3 to DN2 thymocytes. While one would expect the presence of  $V_{\beta}$ - $D_{\beta}$ - $J_{\beta}$  rearrangements in DN2-like cells if they were derived from the more mature DN3 subset, our analysis of  $V_{\beta}$ - $D_{\beta}$ - $J_{\beta}$  recombination is inconclusive due to the fact that a mixed population of DN2 and DN3 cells is present following *ex vivo* DKO culture. Notably, the occurrence of phenotypic reversion in DKO *ex vivo* culture seems to be IL-7 dependent, as we do not see the phenotypic reversion in non-IL-7-containing cultures nor *in vivo*, which may be attributed to IL-7 availability or an unidentified role for thymic stroma in regulating T cell differentiation.

To investigate the influence of stromal cells and Notch signaling on DKO thymocyte phenotypic reversion, we employed the OP9-DL1 culture system. Notch is an important regulator of T cell development and his system has been shown to support T cell differentiation (Schmitt and Zuniga-Pflucker, 2002). While the coculture of DKO thymocytes with OP9 cells does not alter reverse differentiation, coculture with OP9-DL1 stromal cells blocks the DN3 to DN2 phenotypic shift. Notch signaling is important at multiple stages of T cell development and has been implicated in many aspects of cell fate specification including T/B cell-,  $\alpha\beta/\gamma\delta$ -, and CD4/8-lineage choice (reviewed by MacDonald et al., 2001). However, the finding that Notch prevents the backwards

differentiation of E protein deficient thymocytes imparts a novel role for Notch signaling in T cell development. Although the mechanism by which Notch functions in this context is unknown, an interaction of Notch signaling and E protein function has been identified during T cell development. Signaling through the Notch pathway has been shown to inhibit E protein activity (Izon et al., 2002; Ordentlich et al., 1998). In addition, a number of genes that are regulated by Notch signaling are also responsive to E2A (Ikawa et al., 2006). This suggests Notch and E proteins act in parallel to drive T cell differentiation.

A role for E2A in suppressing DN3 cell proliferation has been observed by Engel and Murre in the analysis of E47 deficient mice (Engel and Murre, 2004). However, the signaling events responsible for cell cycle control that are mediated by E proteins at this early stage of T cell development are unknown. Previous studies have implicated E2A in cell cycle regulation by activating CDKIs such as p21<sup>Cip1</sup> in cultured fibroblasts, osteoblasts, and thymoma cells (Funato et al., 2001; Pagliuca et al., 2000; Prabhu et al., 1997; Schwartz et al., 2006). While we observed a decrease in p21<sup>Cip1</sup> expression in DKO thymocytes, no changes in the proliferative status of DN thymocytes from p21<sup>Cip1</sup> deficient mice were detected *in vivo* or *ex vivo* in the presence of IL-7.

In agreement with these findings, defects in T cell development have not been reported in the study of p21<sup>Cip1</sup> deficient mice (Deng et al., 1995). In contrast, gene knockout studies have indicated that p18<sup>Ink4c</sup> and p27<sup>Kip1</sup> appear to play important roles in thymocyte development, as disruption of either p18<sup>Ink4c</sup> (Franklin et al., 1998) or p27<sup>Kip1</sup> (Nakayama et al., 1996) leads to increased numbers of thymocytes. Our analysis showed

that p18<sup>Ink4c</sup> is downregulated whereas p27<sup>Kip1</sup> is up-regulated upon the deletion of E2A and HEB. Transcriptional up-regulation of p27<sup>Kip1</sup> is somewhat surprising since we observed an overall increase of cell cycle proliferation in DKO mice. It is possible that even though p27<sup>Kip1</sup> expression was increased, it was not sufficient to overcome the dramatic decreases of p18<sup>Ink4c</sup> and p21<sup>Cip1</sup>, as the decision to proliferate or arrest is ultimately regulated by the balance of factors that inhibit vs. those that promote cell cycle. Also possible is that enhanced p27<sup>Kip1</sup> expression can activate cyclin D-dependent kinases (Sherr and Roberts, 1999) and therefore promote cell cycle. Finally, the observed increase p27<sup>Kip1</sup> in expression may not be representative of protein levels, as p27<sup>Kip1</sup> activity is regulated at the posttranslational level (Pagano et al., 1995).

We found cyclin D3, cyclin B, and cyclin E expression to be decreased in HEB/E2A-deficient cells. Although we observe a decrease in both DN3 and DN4 cells, the latter display a slightly greater downregulation. While the expression levels and role(s) of cyclins B and E are poorly defined in T cells, cyclin D3 has been shown to influence thymocyte development (Aifantis et al., 2006). In agreement with our results, Song et al. (Song et al., 2004) found E2A to be recruited to the cyclin D3 promoter and activate transcription in a B cell precursor cell line. Similarly, other authors have found decreased expression of cyclin D3 in B- and non-lymphoid cells in the absence of E2A (Zhao et al., 2001). The reduction in cyclins B1 and E is in general agreement with other studies showing a reduction in cyclin expression in E2A-deficient cells (Greenbaum et al., 2004; Zhao et al., 2001). Together these results indicate that the mechanism by which E proteins regulate cell cycle gene expression and proliferation is complex. Accordingly,

it is unlikely that a single gene product is responsible for alterations in E protein-induced proliferation. Rather, it is more likely that a number of cell cycle regulators interact in concert to influence cell cycle.

Our analysis of p18<sup>Ink4c</sup> knockout mice provides an important clue as to how E proteins may participate in cell cycle regulation during early T cell development. The increased proliferation seen in p18<sup>Ink4c</sup> deficient DN thymocytes even in the presence of E protein function suggests that p18<sup>Ink4c</sup> may act downstream of E proteins. In fact, the observed decrease of p18<sup>Ink4c</sup> expression in DKO thymocytes further implicates p18<sup>Ink4c</sup> as a downstream effector molecule of E protein signaling that bridges their function to cell cycle regulation. Based on our findings, it is unclear how E proteins regulate p18<sup>Ink4c</sup> expression. We did not detect any direct transactivation of the p18<sup>Ink4c</sup> promoter by E2A in transient transfection reporter assays. Although the transcription factor menin (*Men1*) was slightly reduced in DN3 DKO thymocytes, it is unlikely that this mild alteration is solely responsible for the dramatic increase in DN3 DKO thymocytes.

Analysis of signaling molecules associated with IL-7 similarly provided little insight into the mechanism(s) by which disruption of E proteins may advance cell cycle progression. All of the genes analyzed showed only slight alterations in expression following the disruption of E protein function. While the changes in Jak3, Pten, and FOXO1 are consistent with the increased proliferation of DKO thymocytes, it remains to be determined whether these mild differences manifest any physiological consequences. Although IL-7R $\alpha$  has been shown to be a target of E proteins, we did not observe any changes in expression in DKO thymocytes. This is likely due to the fact that expression

levels of IL-7R $\alpha$  are already significantly decreased by the time *Lck*-Cre is active to delete E2A and HEB (Sudo et al., 1993).

The *ex vivo* culture of DN cells was carried out in the presence of IL-7 as the sole cytokine and in the absence of stromal cells. IL-7 has been shown to provide both survival and proliferation signals during early T cell development. Thus, the observed increase in proliferation of DKO and p18<sup>Ink4c</sup> deficient thymocytes suggests that E proteins provide a critical counter-balance to suppress IL-7 mediated proliferation in developing T cells undergoing TCR $\beta$  gene rearrangement. In addition to IL-7 signaling, Notch has been shown to provide an essential signal for early thymocyte development (Robey and Bluestone, 2004). However, the absence of stromal cells in our culture system suggests that proliferation of DKO thymocytes occurs independent of Notch signaling.



**CHAPTER 5**  
**CONCLUSIONS AND FUTURE DIRECTIONS**

In summary, our findings suggest that E proteins are important regulators of T cell differentiation, proliferation, and apoptosis. The role for E proteins in lymphocyte development has been underappreciated due to the presence of significant functional redundancy among the different E proteins. Such function overlap has obscured the importance of E proteins as evidenced by the relatively mild phenotype of single gene knockouts. In contrast to *Drosophila*, which only contains a single E protein gene *Daughterless*, mammals contain three: E2A (which encodes the protein products E12 and E47 generated by alternative splicing), HEB, and E2-2. The presumed rationale for encoding multiple E proteins is to accommodate the increased complexity of the mammalian organism and thereby provide precise regulation of gene expression in a tissue- and stage-specific manner (Zhuang et al., 1998). This fine-tuned regulation of E proteins during T cell development is manifested by the distinct expression patterns of HEB and E2A as well as the observation that particular E boxes appear to preferentially bind particular E protein dimer pairs (Hu et al., 1992; Lassar et al., 1991; Sun and Baltimore, 1991).

E2A and HEB are believed to be the two most important E proteins in T cells and function as heterodimers (Sawada and Littman, 1993; Shen and Kadesch, 1995). Importantly, these two E proteins are activated at different points during T cell development, with E2A expression being turned on earlier than HEB (Engel et al., 2001; Pan et al., 2002; Taghon et al., 2006). The functional consequences of distinct expression patterns is reflected by the DN1 vs. ISP block in thymocyte development resulting from

the disruption of E2A or HEB, respectively (Barndt et al., 1999; Zhuang et al., 1994). As such, it is possible that E2A:E2A homodimers are functionally active during early stages of DN development and only upon further progression do E2A:HEB heterodimers become active (Pan et al., 2002). This is supported by the lack of any early DN defect associated with the loss of HEB. Alternatively, there may be particular targets or functions of E2A during early DN thymocyte development that cannot be substituted for by HEB. However, this is unlikely based on the finding that in the absence of a particular E protein, remaining E proteins are typically able to functionally compensate for the loss by directing the transcription of gene targets normally regulated by the missing E protein. The occurrence of this phenomenon is supported by both molecular and genetic evidence. Examination of E protein binding to a canonical T cell E box site normally occupied by HEB:E2A heterodimers showed that in the absence of HEB, E2A:E2A homodimers could bind the E box site. The converse situation also occurs such that the loss of E2A allowed for HEB:HEB homodimer formation and binding to the E box (Barndt et al., 2000).

Whether all regulatory E box sites are capable of being bound by different E protein pairs remains unknown. Affinity studies performed on E box sites clearly indicate a preference for particular bHLH protein dimers (Beltran et al., 2005). However, when the preferred binding dimer is absent, affinity-based competition for binding is alleviated and the remaining dimer pair may be able to bind the E box and regulate target gene transcription. Genetic support for functional redundancy between E2A and HEB is demonstrated in locus exchange experiments in which the cDNA of HEB was inserted

into the E2A genomic locus (Zhuang et al., 1998). In contrast to E2A knockout mice that display a severe block in B cell development before immunoglobulin rearrangement, B cell development in E2A<sup>HEB</sup> mice was found to be relatively normal even though E2A was disrupted. This suggests that HEB can substitute for E2A to promote developmental progression in B cells and that the targets of E2A can be regulated by HEB. In addition, this suggests that the dosage of E proteins rather than the specific type of E protein is more important in the regulation of lymphoid-specific genes.

Although such compensation is functionally significant, limitations on the ability of E proteins to compensate for one another seem to exist. Supporting evidence of a constraint in E protein redundancy is provided by the finding that B cell development in E2A<sup>HEB</sup> mice is not completely normal (Zhuang et al., 1998). Importantly, structural and functional conservation among E proteins is limited to the bHLH and transactivation domains. Thus there may be unique features outside these conserved sequences that allow individual E proteins to perform specific functions via these non-conserved regions, possibly through interactions with other transcription factors. Also, while E2A mice transcribe HEB with the same kinetics and amplitude as E2A, the post-transcriptional and post-translational regulation of HEB may be different than E2A and therefore mRNA levels would not reflect protein levels of HEB. E2A mRNA levels in different stage T cells are relatively constant while protein levels fluctuate widely (Engel et al., 2001; Pan et al., 2002; Taghon et al., 2006). This is in contrast to HEB mRNA expression that varies during T cell development (Taghon et al., 2006). Thus it appears that the stage-specific expression level of E proteins is the salient feature of E protein

target regulation, although sequence specificity and the ability to interact with other cofactors may certainly contribute to target preferences.

To examine target gene regulation more closely, microarray analysis of the conditional HEB and E2A, and HEB-E2A double conditional knockouts using the same *Lck-Cre* transgene would be beneficial. Comparison of the results obtained via this analysis would reveal the similarity and overlap of targets disrupted by the loss of E2A and/or HEB. Because E proteins are active in a stage-specific manner and *Lck-Cre*-mediated deletion is incomplete at early DN stages, sorted DN3 and DN4 cells would be necessary to obtain and interpret the relevant data. Another application of microarray analysis is the identification of shared vs. unique transcriptional targets of E protein activation. We would predict that E proteins may be involved in the regulation of tissue- and stage-specific genes as well as common targets, e.g. those in the pathway of cell cycle regulation. To these ends, the comparison of E2A target genes in different cell types or distinct stages of T cell development could be beneficial.

Following the disruption of E protein function in our HEB-E2A double conditional knockout, we detect a severe block in thymocyte development at the DN4 to DP transition. We speculate that the severely perturbed expression of pT $\alpha$  in DKO mice is only partially responsible for this block, and that other unidentified roles for E proteins likely contribute to the observed defect, as both the introduction of a TCR transgene and  $\alpha$ -CD3 $\epsilon$  treatment of HEB<sup>ko</sup> or HEB<sup>bm</sup> mice is unable to drive differentiation to the DP stage of development (Barndt et al., 1999; Barndt et al., 2000). The observation of a DN4 to DP block in DKO mice is uncommon. Because signaling through the pre-TCR is

responsible for differentiation to the DN4 and DP stages of development, disruption of any participant involved in pre-TCR expression or signaling results in a DN3 block (Fehling et al., 1995; Mombaerts et al., 1992a; Mombaerts et al., 1992b).

E2A has been identified as being downstream of the pre-TCR (Bain et al., 2001). Signaling through the pre-TCR complex downregulates E2A expression, which appears to be necessary for progression from the DN to DP stage transition. Interestingly, the T cell developmental block present in mice deficient in pre-TCR signaling is rescued in the absence of E2A (Engel and Murre, 2004). While DKO mice are deficient in pre-TCR signaling due to a severe disruption in pT $\alpha$  expression, we do not observe progression to the DP stage of development. The discrepancy between this study and our results implies that a small amount of E proteins is necessary to drive the DN to DP transition.

Mutations that generate a block at the ISP stage of T cell development are also rare. Aside from HEB, TCF-1 and ROR $\gamma$  are examples of the few transcription factors that result in an ISP stage block (Kurebayashi et al., 2000; Sun et al., 2000; Verbeek et al., 1995). It would therefore be reasonable to predict that E proteins may collaborate with transcription factors such as TCF and/or ROR $\gamma$  in the regulation of the ISP stage transition. Preliminary data from our laboratory suggests that E2A is upstream of ROR $\gamma$  (Y. Zhuang unpublished results). Together these results suggest that the heterodimer pair of HEB and E2A perform critical uncharacterized functions during the DN to DP stage of T cell development.

In agreement with other studies suggesting a role for E proteins in cell survival, we detect an increase in apoptosis of DN cells from DKO mice. Specifically, we observe

increased apoptosis of DN3 cells. This may be due to the lack of pre-TCR signaling, which is required for survival of DN3 cells, or alternatively the induction of proliferation during VDJ recombination. If this proliferation occurs during recombination, the double-stranded breaks induced by TCR $\beta$  rearrangement may be interpreted as DNA damage and cause the cell to undergo apoptosis (Kim et al., 1999). Because we observe an increase in the proliferation of DN3 thymocytes from DKO mice, this mechanism warrants further investigation. Additional experimental approaches may be sought to determine whether DNA damage contributes to apoptosis of DKO thymocytes. For example, one could culture DKO cells *ex vivo* in the presence of IL-7 while exposing them to DNA damage-inducing conditions such as ionizing (UV) radiation. In addition to determining the effects of such treatment on apoptosis, we can ask whether proliferation still occurs in these cells or if the DNA damage checkpoint is disrupted. It is possible that E proteins influence the regulation of checkpoint components. Alternatively, E proteins may be downstream of the DNA damage checkpoint and their disruption prevents efficient execution of the apoptotic signaling pathway and allows aberrant proliferation in the presence of DNA damage.

Importantly, the results of our work imply a critical role for E proteins in the coordination of differentiation and proliferation events prior to the beta selection checkpoint. In the absence of E proteins we describe the novel finding of reverse phenotypic differentiation of T cells from the DN3 to the DN2 stage of development. The classification of DN2 vs. DN3 cells can be made using a number of developmental, functional, and phenotypic criteria. The status of the TCR $\beta$  chain rearrangement is the

salient feature of T cell development. During the CD44<sup>+</sup>CD25<sup>+</sup> DN2 stage of development, thymocytes undergo a transient burst of proliferation followed by D<sub>β</sub>-J<sub>β</sub> recombination. Once cells reach the CD44<sup>-</sup>CD25<sup>+</sup> DN3 stage, cells become quiescent and V<sub>β</sub>-D<sub>β</sub>J<sub>β</sub> rearrangement occurs. Upon the completion of recombination, the TCRβ chain is expressed on the cell surface in conjunction with pTα to form the pre-TCR. Another important difference between these cells is that DN2 cells are responsive to the proliferation-inducing effects of IL-7, while DN3 cells are unresponsive and proliferate only following pre-TCR signaling (Di Santo et al., 1999; Trop et al., 2001).

The molecular nature of the DN2-like cells following the reverse phenotypic reversion of DN3 DKO thymocytes *ex vivo* in the presence of IL-7 remains unknown and warrants further investigation. It is unclear whether these cells assimilate legitimate DN2 functional characteristics or merely acquire the phenotypic markers of DN2 cells. In our analysis of VDJ recombination of the TCRβ chain following *ex vivo* culture, we observe the presence of V<sub>β</sub>-D<sub>β</sub>J<sub>β</sub> rearrangement products suggesting that these cells derive from DN3 cells. However, these results are inconclusive since both DN2 and DN3 cells are present at the time of harvesting. To assess the recombination status of the DN2 population, post-culture sorting of DN2 and DN3 cells should be independently analyzed for the presence of TCRβ rearrangement. Importantly, the assessment of TCRβ rearrangement is not a suitable means for determining the DN2 vs. DN3 status of DKO thymocytes because if the DN2 cells are derived from DN3 cells, they will contain V<sub>β</sub>-D<sub>β</sub>J<sub>β</sub> rearrangements. A final difference between DN2 and DN3 cells is their ability to undergo alternative lineage development. In WT mice, DN2 cells are not restricted to the



T cell lineage and are able to give rise to NK and dendritic cells (Godfrey et al., 1994; Moore and Zlotnik, 1995; Wu et al., 1996). In contrast, DN3 cells are committed to the T lineage (Fehling et al., 1995; Godfrey et al., 1994; Godfrey et al., 1993). To test whether the observed DN2-like reversion cells are actually DN2 in terms of their differentiation capacity, DN3 thymocytes from DKO mice can be sorted and placed in culture in the presence of IL-7. Following the characteristic reverse phenotypic differentiation, IL-15 could be added to the culture in place of IL-7. IL-15 has been well-characterized for its ability to drive NK cell development (Mrozek et al., 1996; Waldmann and Tagaya, 1999). The development of NK cells could be measured by phenotype analysis of surface markers such as DX5 as well as functional killing assays. If DKO thymocytes can undergo NK cell differentiation, it is likely that these cells are bona-fide DN2 cells in terms of their lineage plasticity. If we do not observe such a change, the DN2-like population may merely represent a phenotypic reversion.

To test the ability of these DN2-like cells to undergo alternative lineage differentiation *in vivo*, DKO thymocytes cultured *ex vivo* in the presence of IL-7 could be labeled and transferred to recipient mice by intrathymic injection. As a corollary experiment, one may label DKO DN3 thymocytes and perform intrathymic injection and determine whether any DN2 phenotypic reversion occurs. Although we observe this phenomenon *ex vivo*, it is possible that the thymic environment will inhibit the ability of E2A-HEB deficient thymocytes to undergo DN2 phenotypic reversion.

Interestingly, culture of DKO thymocytes on OP9 and OP9/DL1 stromal cells revealed that the phenotypic dedifferentiation of DN3 DKO thymocytes is prevented by

Notch signaling. This finding indicates a novel role for Notch signaling in the context of E protein deficiency such that Notch activity can overcome the phenotypic reversion defect caused by the loss of E proteins. If in fact these cells are found to undergo genuine backwards differentiation and not just phenotypic reversion, this implicates a role for Notch in enforcing the developmental fate of T cells. Our results further demonstrate that Notch signaling cannot substitute for E protein function during the transition from the DN to DP stage of differentiation. DKO thymocytes cultured *ex vivo* on OP9-DL1 stromal cells do not progress to the DP stage of development and remain in the DN compartment.

To verify the involvement of Notch signaling in these cultures, we can measure the expression of Notch signaling components or downstream targets. For instance, because Notch is cleaved from the membrane during activation, levels of intracellular Notch could be measured by Western blot. Similarly, the expression of Notch targets could be determined by real time PCR. Also, to ensure that the effects of the OP9-DL1 culture are specific to Notch signaling,  $\gamma$ -secretase inhibitors could be used to block the cleavage of Notch from the cell membrane (Geling et al., 2002; Hadland et al., 2001). By extending this approach to an animal model, it would be interesting to test whether administration of  $\gamma$ -secretase inhibitors to DKO mice can cause phenotypic reversion *in vivo*.

An interaction between Notch signaling and E protein function has been established. Notch has been shown to downregulate E2A activity by targeting it for ubiquitination and subsequent degradation (Huang et al., 2004; Nie et al., 2003;

Ordentlich et al., 1998). A more recent study has suggested that E2A and Notch appear to function in separate as well as partially overlapping pathways to regulate T cell differentiation (Ikawa et al., 2006). The *ex vivo* culture system we have established using DKO thymocytes provides a unique opportunity for the further dissection of these two important pathways. For example, both Notch and E2A have been shown to regulate expression of HES1, a transcription factor involved in DN thymocyte development (Ikawa et al., 2006). However, it is not known whether Notch mediated HES1 activation is dependent on functional E proteins. Furthermore, if HES1 expression is absent in DKO cells cultured on OP9/DL1 stromal cells, it would be interesting to determine whether ectopic expression of HES1 is sufficient to drive development from the DN to DP stage of T cell development.

The results of our HEB and E2A deletion status analysis from the DKO *ex vivo* cultures are inconclusive. Because we do not see complete phenotypic reversion of all DKO thymocytes in the presence of IL-7, a mixed population of cells exists that likely has only partially deleted their E protein alleles. Since E proteins have been implicated in regulating cell death, it is possible that those cells which have undergone phenotypic reversion contain only partial E protein deletion and cells which have undergone complete E protein deletion die by apoptosis. This could also potentially explain why we don't observe the expected amount of E protein deletion in our cultured cells. This would insinuate that different threshold levels of E proteins are present in regards to their functional role in differentiation, apoptosis, and proliferation. To test this hypothesis and more closely examine dosage effect of E proteins on the aforementioned biological

processes, one can utilize different HEB and E2A allelic combinations. For example, a comparison of  $HEB^{flox/flox}E2A^{flox/+}Lck-Cre^+$ ,  $HEB^{flox/flox}E2A^{+/+}Lck-Cre^+$ ,  $HEB^{flox/+}E2A^{flox/flox}Lck-Cre^+$ ,  $HEB^{+/+}E2A^{flox/flox}Lck-Cre^+$ , and  $HEB^{flox/+}E2A^{flox/+}Lck-Cre^+$  thymocytes could be placed in culture in the presence of IL-7 and analyzed for their ability to undergo reverse phenotypic reversion, proliferation, and apoptosis.

Our studies further suggest a previously unidentified role for E proteins in suppressing IL-7 mediated proliferation at the DN3 stage of development. The exact mechanism(s) responsible for E protein-mediated regulation of cell cycle in DN thymocytes is unknown and requires further investigation. The regulation of cell cycle control gene expression by E proteins is likely to be complex. This is supported by our findings that E proteins appear to positively regulate the expression of both pro- and anti-cell cycle progression genes such as cyclins and CDKIs, respectively. The decrease of both cyclin and CDKI expression in E2A-HEB deficient thymocytes does not provide a simple explanation for the mechanism of E protein-mediated cell cycle progression. Since the decision to advance or arrest cell cycle is ultimately determined by the balance of pro- vs. inhibitory factors, it is possible that the observed decrease in  $p18^{Ink4c}$  simply overwhelms the decrease in cyclins and proliferation ensues. However,  $p18^{Ink4c}$  is most likely only one of many targets regulated by E proteins able to contribute to increased cell proliferation of DKO thymocytes. Accordingly, many unidentified targets of E proteins capable influencing cell cycle presumably exist.

The genetic system reported herein provides a valuable experimental model for identifying and testing additional E protein targets responsible for cell cycle regulation.

Work reported here is also relevant to the observation that E2A deficient mice frequently develop T cell leukemia (Bain et al., 1997; Yan et al., 1997). Studies have suggested that leukemogenesis is linked to the loss of E2A in early stage of T cell development (Pan et al., 2002). Therefore, future research on E protein function in cell cycle progression should impact our understanding of both basic mechanisms of T cell development and leukemogenesis.

To summarize, the results of our studies reveal a number of crucial roles for E proteins in the development, apoptosis, and proliferation of T lymphocytes. The multiple biological roles of E proteins during thymocyte development suggest E proteins are situated in a pivotal position upstream of numerous physiological signaling cascades. These functions are regulated in a tissue- and stage-specific manner as part of the cellular developmental program. As such, E proteins control both lymphocyte-specific and common genes involved in basic cellular processes such as apoptosis and cell cycle, which are unlikely to be unique to T cell development. Our E2A-HEB double conditional knockout thus provides an extremely useful model for studying E proteins due to its ability to alleviate concerns of functional redundancy among different E proteins. In addition, by utilizing a different promoter to drive Cre transgene expression our system can be easily expanded and applied to numerous lymphoid and non-lymphoid cell types and/or stages of development to fully elucidate the many as-yet uncharacterized biological roles of E proteins.

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### PUBLICATIONS

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