Establishment of a working model for the study of HEB transcription factor activity
and elucidation of the function of the alternative domain of HEBAlt

By

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Abstract

T cell development in the thymus occurs in a series of tightly regulated stages and is controlled by a complex network of transcription factors. E proteins, part of the class I basic-helix-loop-helix (bHLH) transcription factors, have been shown to be critical for T cell specification and commitment, as E protein deficiencies leads to major breaches in the T cell developmental program. There are three members of the E protein family: HEB, E2A and E2-2. HEBAlt, the shorter alternative isoform of HEB, has a unique and highly conserved alternative domain, the function of which still remains elusive. I was able to show that HEBAlt is also able to positively regulate the RORγt promoter in the context of 293T cells, and that the alternative domain is only partially responsible for this function. Further research is needed to elucidate the role of HEBAlt in T lymphopoiesis and its interactions with other factors.
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Chapter 1: Introduction and Aims
T cell development

T cells are an essential component of our adaptive immune system. They are able to recognize foreign antigen and mount an attack to prevent infection. Thymocytes undergo a complex and tightly regulated developmental process in the thymus, and dysregulation of the network leads to breaches in immunity, such as cancer, autoimmunity, and immunodeficiencies. T cell development begins when hematopoietic stem cells migrate from the bone marrow to the thymus. These thymic seeding progenitors (TSPs) enter the thymus at the corticomedullary junction (CMJ)(1). The most immature thymocytes are called double negative (DN) cells because they lack the expression of both the CD4 and CD8 co-receptors (1). There are four DN stages: DN1 to DN4 (Figure 1). DN1 cells include ETP (early T cell progenitors), a highly heterogeneous population that still retain NK (natural killer), DC (dendritic cell) and myeloid potential (1). ETPs are CD44+CD117+CD25-. The DN1 to DN2 transition coincides with specification, which is when the genes specific for the T lineage are turned on (2). DN2 cells upregulate CD25 (IL-2Rα) and CD90 (Thy-1), induce the Rag (recombinase activating gene)-1 and Rag-2 recombinases, upregulate pre-Tα (pTα), start gene rearrangement at the TCRβ/γ/δ gene loci, and turn on the lck tyrosine kinase (3). The αβ and γδ lineage divergence is thought to be completed by the DN3 stage. Entry into the DN3 stage marks commitment, which is the loss of alternative lineage potential and commitment to the T cell fate. DN3 cells downregulate both CD44 and CD117 (3). The first major developmental checkpoint, β-selection, occurs at the DN3 stage and signaling through the pre-TCR is required to proceed to the DN4 stage (2). The pre-TCR complex is made up of successfully rearranged TCRβ chains paired with the invariant pTα chains, along with the CD3 signaling components (2). Pre-TCR signaling leads to a downregulation of CD25, pTα and Rag1/2 (3).
Figure 1. T cell development in the thymus. T cell development proceeds through a series of tightly regulated steps and checkpoints. It starts with the four double negative stages (DN), which are distinguished by the markers CD44 and CD25. At the DN1/2 stage, the cells are still heterogeneous and still have alternative lineage potentials. The DN3 stage (β-selection) is the first major checkpoint, and a pre-TCR signal is required. Then there is an intermediate ISP (immature single positive stage) that is CD8+, followed by the double positive (DP) stage, which is the second developmental checkpoint. After signaling through the αβTCR and positive selection, the thymocytes differentiate into mature single positive (SP) CD4+ or CD8+ T cells. The E proteins E2A and HEBCan are expressed throughout T cell development, while HEBAIt is restricted to the early DN2/3 stages. RORyt is mostly expressed in DPs.
Cells lacking productive TCR\(\beta\) rearrangements or those that do not receive a pre-TCR signal will die by apoptosis (2). DN4 cells are CD44-CD25- and progress into a transient immature single positive (ISP) stage with the upregulation of CD8 (3). The ISPs are distinguished from the mature CD8 single positive (SP) cells by a lack of cell surface TCR\(\beta\) and high expression of CD24 (2). As the cells enter into the double positive (DP) stage, Rag genes are re-expressed and TCR\(\alpha\) gene rearrangements take place (2). The second developmental checkpoint is marked by the formation of the mature TCR\(\alpha\beta\) complex, with TCR\(\alpha\) chains pairing with TCR\(\beta\) chains and the CD3 components (2). After the DP thymocytes undergo positive selection (interact with self-peptides presented by MHC class I and II molecules), they differentiate into either CD4+ or CD8+ SP thymocytes (3). T cells that are potentially autoreactive due to overly strong signals are deleted by negative selection or re-directed into non-classical T cell populations such as T regulatory cells.

**E protein transcription factors**

My focus in this thesis is on the E protein HEBAlt and its interactions with other E proteins. E proteins are a group of transcription factors that play a role in regulating many different immune processes, such as cell survival, cell cycle progression, lymphocyte development and differentiation (4). E proteins belong to the larger family of HLH (basic helix-loop-helix) proteins. There are six different classes of HLH proteins, with E proteins belonging to class I (5). The class I HLH transcription factors are ubiquitously expressed and include HEB (*Tcf12*), E2A (*Tcf3*), E2-2 (*Tcf4*) and the Drosophila homologue Daughterless (5). The class II proteins are tissue-specific and include MyoD, Myogenin and Myf-5 (5). They function in many different developmental systems but are mainly studied in muscle. The three members of the E protein family all share common structural features (**Figure 2**). They all contain a common C-
Figure 2. Structure of E proteins. E proteins belong to the Class 1 HLH (helix-loop-helix) transcription factors. There are three members of this group: HEB (Tcf12), E2A (Tcf3) and E2-2 (Tcf4). All three of them share a common C-terminal bHLH (basic helix-loop-helix) domain, which is responsible for DNA-binding and dimerization. E proteins bind to consensus E-box sites (CANNTG sequence). E proteins function as obligate dimers. They also contain activation domains (ADs) that are responsible for the recruitment of co-activators and co-repressors. Due to alternative splicing and separate transcriptional start sites, there are two isoforms of HEB: the longer canonical HEBCan and the shorter alternative HEBAlt. HEBAlt lacks AD1 and instead has a unique and highly conserved alternative domain. E2-2 also has two isoforms and is paralogous to HEBCan and HEBAlt. The E2A gene encodes for two different proteins (E47 and E12), which differ only in the use of an alternatively spliced exon in the bHLH domain.
terminus bHLH (basic helix-loop-helix) domain, with the basic DNA-binding region and the HLH domain for dimerization (4). E proteins function as obligate dimers and can form either homodimers or heterodimers. They have been shown to bind DNA sequence elements called E boxes, which contain the consensus CANNTG sequence (6). By way of alternative splicing and unique transcriptional start sites, there are two isoforms of HEB: the longer canonical HEBCan and the shorter alternative HEBAlt (7). HEBCan contains two activation domains (AD1/2), which function to recruit different corepressors and coactivators (8). AD1 has been shown to directly recruit the nuclear HAT (histone acetyltransferase) complex SAGA via a highly conserved LDFS motif, and thus suggests that E proteins may play a role in chromatin remodelling through histone modification (6). AD1 and AD2 also cooperatively interact with coactivators such as p300/CBP and GCN5, as well as corepressors from the ETO (eight twenty-one) family (8). HEBAlt lacks AD1 and instead has a unique and highly conserved 23 amino acid alternative domain, the function of which still remains elusive (7). The E2-2 gene locus also encodes two proteins, E2-2Can and E2-2Alt, which are paralogous to HEBCan and HEBAlt. There is only one study published that addresses a possible differential function between these E protein forms, in which E2-2Can was able to inhibit MyoD driven expression whereas E2-2Alt was not (9). In terms of hematopoiesis, E2-2 is known to regulate the development of plasmacytoid dendritic cells (PDCs) and is involved in inducing type I IFN secretion (10). The E2A gene encodes for two different proteins (E12 and E47), but unlike HEB and E2-2, these proteins differ only in the use of an alternatively spliced exon in the bHLH domain (11).

**E2A in lymphocyte development**
E2A has been shown to be crucial for B cell development, as E2A-deficient mice have disrupted development at a very early stage, before the onset of immunoglobulin heavy chain rearrangement (12). It also regulates the initial expression of B-lineage-specific genes, such as EBF1 (early B cell factor 1) and Pax5 (paired box gene 5) (1). E2A is also important for the suppression of alternative non-B cell lineages, such as macrophage development (11). Furthermore, E2A-/ mice have perturbed T cell development, with a partial DN1 block, ten-fold lower thymocyte numbers as well as accelerated transition from the DP to SP stage (13). Mice lacking the E2A gene also display higher frequencies of thymic-derived T cell leukemias from uncontrolled proliferation (13). E2A has also been called a gatekeeper that prevents cells from progressing to the SP stage until a functional αβTCR is produced (14). Therefore, downregulation of E2A is required upon positive selection in order for cells to proceed past the TCR checkpoint to the SP stage of development (14).

Id (inhibitors of DNA binding) factors

Another class of HLH proteins that are of interest to us are the class V proteins, which include Id factors. There are four Id proteins in vertebrates (15) and the Drosophila homologue is called extramachrochaete (emc)(16). Id factors dimerize with other HLH proteins and inhibit the DNA-binding capability of their partner because they lack the basic DNA-binding domain (5). Upon TCR ligation, the rapid induction of the immediate-early growth response gene 1 (EGR1) leads to increased Id3 expression, which then acts to decrease the DNA-binding ability of E2A (16) (Figure 3). Furthermore, the thymi of Id3-/ mice show the aberrant development of T_{FH} (follicular helper T cells)-like cells that support the proliferation of B cells and germinal center formation as well as the presence of B cell follicles (17). Id2 is essential for the development of mature natural killer (NK) cells as well as the development of lymphoid tissue-inducing (LTi)
Figure 3. Network schematic of the interactions between different transcription factors during T cell development. Delta-Notch signaling is required for TCR ligation. Pre-TCR signaling in DN3 cells induces the upregulation of Egr1/3 (immediate-early growth response genes), which leads to increase in Id3 expression, which suppresses the activity of E proteins. HEBCan and Notch signaling act synergistically to upregulate the transcription of HEBAlt. HEBAlt also upregulates Notch1 in a positive feedback loop. HEB is also able to upregulate Gfi1b, which is a repressor of Gata3 and downregulate Gata3 expression. HEB can also positively regulate the transcription of RORγt. Bcl11b is a downstream target of Notch1 and prevents alternative lineage (ie NK cell) development by repressing Id2 expression.
cells that are required for the formation of secondary lymphoid organs such as lymph nodes and Peyer’s patches (PP)(18). The disruption of the Id2 genes results in a block in NK cell and NKT cell development (4).

**HEB factors in T cell development**

HEB (HeLa E-box binding) factors are known to be critical regulators of lymphocyte development. They were first discovered in 1992 during the isolation of a cDNA library in search of factors that bind to enhancer sequences in immunoglobulin genes (19). HEBAlt was discovered in 1999 when screening a cDNA library constructed from SCID (severe combined immunodeficiency) thymocytes for transcription factors involved in T cell specification (20). HEBCan is expressed throughout T cell development, with peak mRNA expression at the DP stage of development, whereas HEBAlt is induced in early T cell precursors and permanently downregulated at the DN to DP transition (7) (Figure 1). HEBAlt is required for the generation of early T cell precursors, as its expression coincides with the induction of pro-T cell genes such as Rag1/2 and pTα (7). The retroviral transduction of HEBAlt into fetal liver LSK cells developing on OP9-DL1 co-cultures led to an increase in the number of T cell precursors, whereas the sustained expression of HEBCan did not alter the number of DP cells but was inhibitory for proliferation (7). Since the HEB null mutation is embryonic lethal on the C57Bl/6 background, all studies had to be conducted with fetal samples. Since HEB-/- mice were generated by deleting a segment of the bHLH domain, these mice lack both HEBCan and HEBAlt (2). The loss of HEB resulted in a partial block at the DN3 stage as well as the ISP to DP transition, and caused an accumulation of ISP cells and a decreased percentage of DP cells and overall thymic hypocellularity (7). Data from our lab has also shown that HEB-/- DN3 cells have a defect in T cell commitment, adopt a DN1-like phenotype and can be induced to
differentiate into functional thymic NK cells (tNK) in vitro by culturing on OP9-GFP stromal cells with IL-7 and IL-15 (21).

**HEBAlt in hematopoiesis**

Studies of HEBAlt have been facilitated by the creation of mice in which the transgenic expression of HEBAlt is driven by the lck proximal promoter, leading to expression of HEBAlt at the DN2 stage. Transgenic expression of HEBAlt in HEB-/- precursors was able to partially rescue the cells from the delay in T cell specification in fetal thymic organ cultures (FTOCs) (7). HEB factors directly upregulate pTα by binding to the series of E-box sites present in the pTα promoter and enhancer region (22). Results from our lab have also shown that the transgenic expression of HEBAlt was not able to overcome the block in β-selection caused by Rag deficiency in the Rag-1/- mice. Since E2A deficiency does lead to a breakthrough to the DP stage (14), these data imply that HEBAlt does not act at this stage by inhibiting the function of E2A-containing dimers. Interestingly, HEB-/- Rag-1/- precursors showed impaired expression of pTα and CD3ε that can be rescued by the introduction of the HEBAlt transgene in the FTOC system, suggesting that HEBAlt could be a direct regulator of these genes at the DN stage of T cell development (23). In earlier studies by another group, the defects in HEB-/- thymocytes could not be rescued in adult mice by either injection of anti-CD3 or reconstitution with a TCRαβ transgene. Therefore, it was proposed that HEB functions in parallel with pre-TCR signaling (24). Combined, these studies suggest that HEBAlt and HEBCan are both required for progress through T cell development.

HEBAlt functions in a highly context-dependent way, as it requires DL-Notch signaling to inhibit myeloid development but is able to inhibit B cell development in a Notch-independent
manner (25). It has also been shown that HEBCan and Delta-Notch signaling act synergistically to upregulate the transcription of HEBAlt (7). HEBAlt is also able to upregulate Ikaros and Notch1 in a positive feedback loop and downregulate the myeloid transcription factor C/EBPα (26). The literature also suggests that HEB is able to upregulate Gfi1b, which is a repressor of Gata3, and thus downregulates Gata3 indirectly (21)(Figure 3). HEBAlt functions in parallel with Bcl11b during T cell development and Bcl11b is able to suppress NK fate by inhibiting Id2 expression (2).

The functions of RORγt

RORγt is another transcription factor that has been shown to play a vital role in T cell development. RORγ (retinoic acid receptor-related orphan receptor) is an orphan nuclear receptor that is important for thymocyte differentiation and lymphoid organ development (27). RORγt is the thymus-specific isoform of RORγ that is highly expressed in CD4+CD8+ DP thymocytes, downregulated after positive selection and turned off in mature SP thymocytes (27). Nuclear hormone receptors consist of more than 60 members and share four common structural domains: N-terminal domain (transactivation), central DNA-binding domain (DBD), hinge domain and the C-terminal ligand-binding domain (LBD) (27). The downregulation of RORγt is needed for thymocyte maturation, since the transgenic expression of RORγt inhibits T cell proliferation and inhibits the upregulation of TCR expression in mature T cells (28). RORγt is also important for promoting DP thymocyte survival via the induction of the anti-apoptotic protein Bcl-xL (27), as well as inhibiting cell division by activating the murine cytoplasmic polyadenylation element binding protein 4 (mCPEB4)(29). Furthermore, RORγt also inhibits IL-2 production in developing thymocytes by negatively regulating the transcription of c-Rel (27). RORγt is also vital for the development of lymph nodes and Peyer’s patches (PP)(27). RORγt-
deficient mice have DP thymocytes with increased cell cycle activity and poor survival, and these mice also have abnormally small thymi and no lymph nodes (29). Furthermore, RORγt is the main factor controlling IL-17 transcription and differentiation of lymphocytes that produce IL-17 (30).

**Interplay between RORγt and E proteins**

During T cell development, RORγt is involved in a regulatory network with Egr3 (early growth response gene 3) and E proteins in mediating the effects of pre-TCR signaling (29). Egr3 is involved in delaying the induction of RORγt following pre-TCR signaling to allow for a period of proliferation (29). Following pre-TCR signaling, Egr3 upregulates the E protein inhibitor Id3 and inhibits RORγt expression (29). The RORγt promoter region contains several conserved consensus E-box sites that have been shown to recruit E proteins (29). Current research has shown that E proteins are able to positively regulate the transcription of RORγt, as major deletions of HEB and E2A lead to reduced RORγt expression (30). Zhang et al showed that co-transfection of either E2A or HEB with a RORγt-pGL4 promoter-reporter construct led to increased signal in a dual luciferase reporter assay compared to the empty vector control in EL4 cells (30). Furthermore, chromatin immunoprecipitation (CHIP) assays showed that E proteins were able to directly bind to the RORγt promoter in the context of Th17 cells (30). Therefore, it is evident that RORγt is a direct target of E proteins.

**Innate lymphoid cells (ILCs)**

Innate lymphoid cells (ILCs) are a group of Rag-independent lymphocytes that play important roles in innate immunity (31). They have a wide variety of functions, including maintaining epithelial homeostasis, wound healing, intestinal and mucosal defense, lymphoid
tissue generation as well as tissue remodeling and repair (32). ILCs are defined as cells that lack functionally rearranged T-cell receptor (TCR) or immunoglobulin (Ig) genes (31). They are also dependent on Id2 expression for differentiation and maintenance, and also require IL-7Rα signaling (32). Furthermore, they require common cytokine receptor γ-chain (IL-2Rγ) for development (33). ILCs are categorized into three distinct groups: ILC1, ILC2 and ILC3. The Group-1 ILCs include natural killer (NK) cells that produce IFN-γ in a Th1 cell-like response (33). The development and function of ILC1 is heavily regulated by the transcription factors T-bet (Tbx21) and Eomes (Eomesodermin)(33). The Group-2 ILCs are also known as natural helper cells, innate helper 2 (Ih2) cells or nuocytes, and they produce a Th2 cell-like response, with the production of IL-4, IL-5, IL-9 and IL-13 (33). They are responsive to IL-25 and IL-33 (34). ILC2 depend on the transcription factors Gata3 and RORα for development and play important roles in allergic responses (33). The Group-3 ILCs include lymphoid tissue inducer (LTi) cells and produce IL-17 (ILC17) or IL-22 (ILC22, NK22, NCR22)(33). ILC3 cells elicit a Th17/Th22-like response, signal through the aryl hydrocarbon receptor (Ahr), and LTi help induce the formation of lymph tissues (lymph nodes, Peyer’s patches) during embryogenesis (35). ILC17 and ILC22 play a protective role during intestinal infections and inflammation and their dysregulation leads to inflammatory bowel diseases (IBDs) and other autoimmune diseases (35). ILC3 are dependent on the transcription factor RORγt (encoded by Rorc gene) for their development (33).

**MNK-3 cells as an in vitro model of ILC-3 function**

The MNK-3 cell line is a novel model cell line for the study of ILC function and differentiation that was generated by Dr. David Allan and Dr. James Carlyle at Sunnybrook Research Institute (31). It displays characteristics that are related to the ILC3 group, such as high
levels of expression of RORγt, Id2, CD127 (IL-7Ra) and IL-2Rγ (31). MNK-3 also express high levels of lymphotoxin α1β2 (LTα/β), which are members of the TNFR (tumor necrosis factor receptor) superfamily and upregulates adhesion molecules ICAM-1 and VCAM-1 (31). MNK-3 can also produce high levels of IL-22 in response to IL-23 and IL-1β stimulation (31). Furthermore, high level of IL-17A production can be induced in this cell line through stimulation with both cytokines (IL-1β, IL-2, IL-23) and mitogens (PMA/ionomycin) (31). They are responsive to gamma chain cytokines and can be differentiated in vitro in the presence of IL-7 and IL-15 (31). The MNK-3 cell line is derived from NKR-P1B+ (inhibitory NK receptor that recognizes Clr-b ligand) NIH-Swiss mouse E15 fetal thymocytes that were co-cultured with Bcl2-retrovirus producer cells and then transfected with SV40 large T antigen and human c-myc (31).

**Dual luciferase reporter (DLR) assay for the detection of gene expression**

A major goal of my thesis was to develop an assay to measure HEB activation of gene expression. Genetic reporter systems have many applications, including being a great tool for the study of eukaryotic gene expression. Dual reporter systems have greater experimental accuracy than single reporter systems, due to the measurement of two individual reporter enzymes: the “experimental” firefly reporter and the “control” Renilla reporter. The co-transfected Renilla reporter serves as a baseline to normalize the activity of the experimental vector, thus minimizing experimental variability due to differences in cell viability or transfection efficiency. The pGL4 luciferase reporter vectors are the latest generation of vectors used as the experimental reporter for optimal expression in mammalian cells. The pGL4.10 [luc2] vector used in my experiments contain the synthetic firefly luc2 as well as the Renilla hRluc luciferase genes. In the DLR assay system, the signal from the firefly and Renilla luciferases are measured sequentially.
The firefly luciferase signal is measured by adding Luciferase Assay Reagent II (LARII) (Promega, Madison, Wisconsin). The Stop & Glo reagent quenches the signal from the firefly luciferase and measures the Renilla luciferase activity. The luminescence between firefly and Renilla luciferases can be discriminated because they have different enzyme structures and substrate requirements. Firefly luciferase is 61kDa and catalyzes beetle luciferin. Renilla luciferase is 36kDa and catalyzes coelenterazine. A drawback to using coelenterazine is that it is known to emit low-level autoluminescence in aqueous solution. However, the Passive Lysis Buffer (PLB) that comes with the DLR assay kit (Promega) is formulated to minimize coelenterazine autoluminescence. Plate-reading luminometers like the Synergy H1 Hybrid Reader are equipped with two reagent injectors and automatically dispense the appropriate volume at the right time. After the plate is loaded with equal volumes of each sample, the machine first injects LARII, measures the firefly luciferase activity, injects Stop & Glo and then measures the Renilla luciferase signal. This system allows for testing of regulatory elements to be expressed in different cellular contexts, and to assess the ability of specific transcription factors to activate their expression in those contexts. Thus, we utilized this experimental system to examine the interactions between HEBAlt, HEBCan, and E2A transcription factors in the activation of putative E protein target genes.

Hypothesis and Aims

The T cell developmental program is known to be tightly regulated by transcription factors that work together in complex gene regulatory networks. T cell development is highly context-dependent and the dosage and combination of different regulatory factors is important to consider. HEB and E2A proteins have been shown to be critical transcriptional regulators of T
cell specification and commitment. The dysregulation of E proteins can lead to breaches of immunity (immunodeficiency), tolerance (autoimmunity) or growth control (cancer). In order to better understand the role of E proteins in T cell development, we first need to understand basic E protein function.

**Hypothesis:** HEBAlt is an important E protein that is able to activate gene expression from E-protein responsive promoters during T cell development, and can form homodimers and heterodimers with other E proteins and Id factors. The presence of HEBAlt in the DN stages of T cell development thus increases the diversity of potential E protein dimers in early T cell development.

**Aim 1.** To develop a working system for the measurement of E protein activity. The dual luciferase reporter (DLR) assay system was used to determine the function of HEBAlt in regulating the transcription of potentially E protein-responsive target genes.

**Aim 2.** To define the types of dimers that can form between the E proteins and Id proteins that are co-expressed during early T cell development. Tagged versions of HEB factors were generated in order to study the binding patterns between E proteins in co-immunoprecipitation experiments.

**Aim 3.** To assess which regions of the Alt domain are required for the ability of HEBAlt to activate transcription. Structure-function analysis was conducted with truncation and point mutants of the alternative domain, and the effects on HEBAlt activity were assessed using the dual luciferase reporter assay.
Chapter 2: Methods
METHODS

Cells

EL4 and NIH3T3 cell lines were obtained from American Type Culture Collection through Dr. James Carlyle (Sunnybrook Research Institute, Toronto, Canada). MNK-3 cells were a gift from Dr. David Allan and Dr. James Carlyle (Sunnybrook Research Institute, Toronto, Canada). HEK293T (293T) cells were obtained from Ashton-Trotman Grant from Dr. Michele Anderson’s lab (Sunnybrook Research Institute, Toronto, Canada).

All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5g/L glucose, sodium pyruvate, supplemented with 10% FBS (fetal bovine serum, Gibco), 100 IU penicillin, 100ug/ml streptomycin, 2mM L-glutamine and 100ug/ml gentamicin. 293T cells were passaged every other day. MNK-3 cells were cultured with 10ng/ml of recombinant mouse IL-7 and 10ng/ml of mouse IL-15 and passaged every 3-4 days. The murine cytokines were obtained from PeproTech.

Reporter Constructs

The RORγt-pGL4 2kb promoter-reporter construct was a kind gift from from Dr. Warren Strober (Mucosal Immunity Section, Laboratory of Host Defenses, NIH, Maryland, USA). The pRL-TK Renilla construct was obtained from Christina Kirkham and Dr. James Carlyle (Sunnybrook Research Institute, Toronto, Canada). All plasmids were transformed using One Shot Top10 Chemically Competent E. coli (Invitrogen) and the DNA extracted using PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen).

Expression plasmids

HEBAlt truncation mutants
For generation of the HEBAlt truncation mutants, the WT form of HEBAlt had been previously cloned into the MIGR1 vector backbone. MIGR1 contains a murine stem cell virus (MSCV) promoter upstream of the multiple cloning site, followed by an internal ribosomal entry sequence (IRES) upstream of a GFP coding cassette. The three HEBAlt truncation mutants (HEBTr, HEBAlt-Met2, HEBAlt-Met3) were generated by previous lab members using 5’ primers encoding one of the three naturally occurring methionines within the Alt domain as a start codon, and a common 3’ primer (Table 1). PCR products were cloned into the MIGR1 vector backbone.

**HEBA** lay point mutants

For the generation of the HEBAlt point mutants, I used the wild-type HEBAlt cloned into the MIY vector backbone. MIY contains a murine stem cell virus (MSCV) promoter upstream of the multiple cloning site, followed by an internal ribosomal entry sequence (IRES) upstream of a YFP coding cassette. The three HEBAlt point mutants (tyrosine to phenylalanine) were generated by designing mutagenesis primers (Table 1) and using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) on the MIY-HEBAlt construct.

**Epitope tagged constructs**

HA (hemagglutinin)- and myc-tagged forms of HEBAlt were previously generated in our lab. For cloning the tagged versions of HEBCan, I PCR amplified the coding sequence of HEBCan from the pCMV-HEBCan template (containing a stop codon) using primers containing the restriction enzyme sites Kpn1 and Sal1 (Table 1; from New England Biolabs). Since the HEBCan contains a Kpn1 restriction enzyme site in the middle, I mutated the site to a synonymous codon using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). I ligated
the PCR product into the pCMV-HA and pCMV-Myc vectors using T4 DNA ligase (Invitrogen) and the 2X Rapid Ligation buffer (Promega). All clones were validated by sequencing (The Center for Applied Genomics, Sickkids Toronto).

**pcDNA3.3-TOPO constructs**

In order to clone the HEBAlt mutants into the pcDNA3.3-TOPO vector, specific primers were designed as shown in **Table 1**. Platinum Taq DNA polymerase (Invitrogen) was used for the PCR amplification reactions. The inserts were cloned into the pcDNA3.3 TOPO vector (Invitrogen) following the manufacturer’s instructions. Resulting constructs were all verified by restriction digest with NheI-HF and Xma1, followed by sequencing.

**Transient transfections**

**Lipofectamine**

293T cells were transfected using the Lipofectamine 2000 reagent (Invitrogen). For the lipofection method, 660,000 cells were plated per well of a 6 well plate the afternoon before the transfection in order to achieve 80% confluency on the day of the transfection. For each transfection, the plasmids (2ug in total) were prepared in 250ul of OPTI-MEM serum-free media (Life Technologies). 10ul of Lipofectamine 2000 reagent were added to a separate Eppendorf with 250ul of OPTI-MEM media and incubated for 5min at room temperature. The Lipofectamine 2000 tube was then mixed with the plasmid tube and incubated at room temperature for 20min. The 293T cells to be transfected were given a media change, and the transfection mixture was added dropwise to the cells.

**Nucleofection**

The EL4, NIH3T3 and MNK-3 cells were transfected using the nucleofection method (Amaxa). Buffer L was used for the EL4 cells (from Amaza Cell Line Nucleofector Kit L, Lonza)
in conjunction with program C-009. Buffer R and program U-030 were used for the NIH3T3 cells, and the MNK-3 cells used Buffer V and program X-005. Two million cells were required for EL4 nucleofection and one million cells were required for NIH3T3 and MNK-3 cell nucleofection. After nucleofection, the cells were placed immediately in pre-warmed DMEM media. The MNK-3 cells were cultured with 10ng/ml of mouse IL-7 and 10ng/ml of mouse IL-15.

**Flow cytometry**

To assess efficiency of transfection, the pMAX-GFP construct (Lonza) was transfected into cells and analyzed by flow cytometry 48hrs later. Propidium iodide (PI) staining was used to determine cell viability. Data was collected using a FACS Calibur flow cytometer (BD Biosciences) to visualize the percentage of GFP+ cells compared to an untransfected control.

**Dual luciferase reporter (DLR) assay**

For the transfection, 500ng of the promoter-reporter construct (RORγt-pGL4 2kb), 50ng of the Renilla construct, and 1ug total of the expression construct(s) were added to each well. DLR assays were performed 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). Lysates from the 293T cells were generated by adding 500ul of 1X passive lysis buffer to the 6 well plate and incubated at room temperature and in the dark for 15 min while shaking. After collecting the lysates, 20ul of each sample was added per well to a 96-well plate in technical triplicates. The two reagents required to measure the luciferase activity are the Luciferase Assay Reagent II (LARII, Dual-Luciferase Reporter Assay System from Promega), which measures the firefly luciferase activity and the Stop & Glo Reagent, which measures the Renilla luciferase signal. The light emitted by the firefly and Renilla luciferase were read using a Synergy H1 Hybrid Reader, using the Gen 5.2.01 program.
Co-immunoprecipitation (Co-IP) and Western Blots

Co-IPs were performed to determine whether two proteins interacted with each other within the same complex. The two plasmids of interest were transfected into 293T cells using Lipofectamine 2000. 24 hours after transfection, the 293T cells were harvested using 250μl of Pierce lysis buffer supplemented with 1 protease inhibitor tablet per 10ml. Lysates were incubated on ice for 30min, with vigorous vortexing every 10min. The lysate was then pre-washed with Pierce Protein A/G agarose slurry for 1hr at 4°C, to block non-specific binding. The immunoprecipitation was done at 4°C on a rotator overnight using c-myc monoclonal antibody-agarose beads (Clontech, clone 9E10). The next day, the samples were washed three times with Pierce lysis buffer and the protein concentrations were determined using the Pierce BCA Protein Assay kit (Pierce Biotechnology). Next, 20μg of the lysate was denatured in SDS loading buffer (containing DTT) and heated at 100°C for 5min. Denatured lysates were loaded onto acrylamide gels containing 10% resolving and 4% stacking gels. Size was determined using the PageRuler Plus Prestained Protein ladder (Thermo Scientific, 10 to 250kDa). After running the gel, samples were transferred onto a PVDF membrane (Biorad TransBlot Turbo) by the semi-dry transfer method using the TransBlot Turbo Machine (Biorad). The blot was then blocked overnight at 4°C on a shaker in 1X TBST (Tris-buffered saline with Tween 20 at 0.1% v/v) with 5% skim milk to eliminate non-specific binding. The next day, the blots were probed for 1 hr at RT with primary antibodies in 1X TBST with 5% BSA. Primary antibodies used were the HA-Tag polyclonal antibody (Clontech, raised in rabbit against KLH-conjugated synthetic HA epitope, purified using Protein A, cat # 631207) and anti-TCF3/E2A (Abcam, ab82847, rabbit polyclonal). To detect HEB, the pan anti-HEB antibody (Santa Cruz Biotechnology, A-20, sc-357) was used.
To detect Id2, the anti-Id2 rabbit polyclonal antibody (Santa Cruz Biotechnology, C-20, sc-489) was used. After washing in TBST, the secondary antibody, HRP-Goat Anti-Rabbit (Invitrogen) (in 1X TBST with 5% milk) and added to the blot for 1hr at room temperature. After washing in TBST, the blot was visualized using the Clarity ECL kit (Biorad) and the Fusion Fx Chemiluminescence and Fluorescence Imager (Vilber Lourmat).

**Statistical tests**

For the dual luciferase reporter (DLR) assays, the samples were all loaded in triplicates. The average was taken of the three firefly luciferase readings as well as for the three Renilla luciferase readings. The firefly luciferase average was divided by the Renilla luciferase average. Next, all the samples were normalized to the control sample containing the empty pCMV-HA vector. Statistical significance was calculated using the GraphPad/Prism program. All graphs used the unpaired, parametric, two-tailed t-test that assumes both populations have the same standard deviation.
Chapter 3: Results
Development of an assay for detecting gene expression by HEB factors

My overall goals in these thesis studies were to determine the ability of HEBAlt to activate transcription from E box sites and to dimerize with other bHLH factors. Furthermore, through structure-function analysis, I hoped to elucidate the portion of the alternative domain that is important for its function. In order to measure E protein activity, an assay system needed to be developed first. My experimental approach was adapted from that used by Zhang et al (30), where they were looking at the regulation of the RORγt promoter by the E proteins E2A and HEBCan in the context of Th17 cells. E proteins are known to bind to E-box sites with the consensus CANNTG sequence. RORγt, an orphan nuclear hormone receptor that is highly expressed in double positive (DP) thymocytes, could potentially be regulated by E proteins because of the presence of a series of E-box sites in its 2 kb long promoter region. The RORγt promoter had been cloned by this group into a pGL4.10 [luc2] luciferase reporter plasmid, to create a promoter-reporter construct (30) (Figure 4). I obtained the RORγt-pGL4.10 construct from the Strober lab and attempted to replicate their results as a baseline for my own experiments, so that I could compare the ability of HEBCan and E2A with HEBAlt to activate expression. The Strober experiments utilized EL4 cells, which are derived from a mouse thymoma cell line. They transiently co-transfected HEB or E2A expression plasmids along with the promoter-reporter construct using Amaxa nucleofection into EL4 cells, and measured luciferase activity after 24 hrs. Both E2A and HEBCan were able to drive expression of luciferase in this system, thus providing me with a potential assay to test HEBAlt activity using HEBCan and E2A as positive controls.

Evaluation of cell lines and nucleofection for transient transfection and expression
Figure 4. Experimental setup for the dual luciferase reporter (DLR) assay. In order to determine the effects E proteins have on the RORγt promoter, an experimental system needed to be established to obtain a readout for the results. The approach I used is to do a dual luciferase reporter assay to measure the level of promoter activation by the transcription factors. The luciferase signal is correlated with the ability of the E proteins to regulate RORγt transcription. The RORγt promoter contains a tandem of consensus E box sites. This promoter is cloned upstream of a pGL4.10[luc2] vector in order to create a promoter-reporter construct. Then the construct is co-transfected transiently (using either lipofection or nucleofection) with the E protein expression plasmids into the appropriate cell line (293T, MNK-3, EL4, NIH3T3). 24 or 48 hrs after the transfection, the luciferase signal produced is measured using a Synergy H1 reader. The firefly luciferase measurement is normalized to the renilla luciferase measurement as an internal control. The non-transfected cells are used as a negative control.
I tested several cell lines including MNK3 cells, EL4 cells, NIH3T3 cells for transfection efficiency using the pMAX-GFP construct and Amaza nucleofection with different programs (Table 2). The pCMV-HEBCan-HA construct was used to test for function and the pCMV-HA empty vector was used as a negative control. For my dual luciferase reporter assay, I was only able to see about a 1.5-fold increase in luciferase signal with HEBCan compared to the empty vector for EL4 cells (Figure 5). The MNK-3 cell line seemed to yield more promising results, showing about a 2-fold increase in luciferase signal for HEBCan compared to control. However, subsequent experiments did not yield the same levels of luciferase induction. It is known that Id2 is very highly expressed in the MNK-3 cell line, suggesting that it might inhibit the activity of HEBCan. Although transfection efficiency was high for the NIH3T3 cells, there was little effect of HEBCan on the RORγt promoter in those cells.

**HEBAIt is able to activate the RORγt promoter in the context of 293T cells**

I next used 293T cells as host cells for transfection using lipofectamine, since these cells are known to be highly transfectable and are routinely used for luciferase assays. I co-transfected 293T cells with the RORγt promoter and either the HEBCan, E2A, or HEBAIt expression constructs, using empty vector to set the baseline. My results showed that E proteins are able to positively regulate the transcription of RORγt in this context (Figure 6). Compared to the empty vector control, HEBCan was able to induce a 2.5-fold increase in luciferase signal and E2A induced a 3-fold increase. In the dual luciferase reporter assay, HEBAIt induced a 2-fold increase in luciferase signal compared to the control, indicating that it is able to activate the RORγt promoter just as well as HEBCan does in 293T cells. Importantly, this is the first time that it has been shown experimentally that HEBAIt can activate transcription.

**Investigating the different binding partners among E proteins**
Figure 5. Testing of the positive control for dual luciferase reporter (DLR) assay in three different cell lines. 2x10^6 EL4 cells were transiently transfected using Buffer L (from the Amaxa Cell Line Nucleofector Kit, Lonza) and program C-009 on the Amaxa machine. 500ng of the RORγt-pGL4.10 [luc2] 2kb promoter-reporter construct was transfected, along with 50ng of pRL-thymine kinase (TK renilla) construct and 1ug of the HEBCan expression plasmid. Sample 1 containing the empty pCMV-HA vector was used as a negative control and sample 2 with the pCMV-HEBCan-HA is used as the positive control. Sample 3 does not contain any transfected plasmids and is used to determine the amount of background luciferase activity in the different cell lines. After 48hrs, the cells are harvested using the passive lysis buffer (PLB) from the Promega Dual Luciferase Reporter Assay kit and the luciferase activity is read using the Synergy H1 Hybrid Reader. The program name is Gen 5.2.01. The firefly luciferase values are normalized to the renilla luciferase values (internal control) to minimize experimental variability.
Figure 6. HEBAlt activates the RORγt promoter in 293T cells. 600,000 HEK293T cells are plated in 6 well plates the day before transfected. On the day of transfection, the cells are at an 80% confluence and were transfected using Lipofectamine 2000 (Invitrogen). 500ng of the RORγt-pGL4.10 [luc2] 2kb promoter-reporter construct was transfected, along with 50ng of pRL-thymine kinase (TK renilla) construct and 1ug of either the HEBCan, HEBAlt or E2A expression plasmid. The empty pCMV-HA vector was used as a negative control. The data is a compilation from four independent experiments. An unpaired parametric t-test was performed to check for significance. ** p<0.01  **** p<0.0001 n.s. = non-significant.
Developing a strategy for detecting different HEB isoforms

E proteins are obligate dimers, and bind to each other through their helix-loop-helix domains. All E proteins and Id proteins, including HEBAlt, contain these HLH domains. After determining that HEBAlt and HEBCan factors are both able to regulate the RORγt promoter, I was interested in finding out whether these transcription factors can form homodimers and heterodimers. HEBAlt contains the same HLH domain as is found in HEBCan, and so theoretically it should be able to form dimers with other E proteins and Id proteins. However, it was possible that the Alt domain could impart conformational changes that might mask or alter this ability. Currently, there is only a commercially available antibody that recognizes both HEBCan and HEBAlt. Therefore, in order to differentiate between HEBAlt and HEBCan, I cloned epitope tagged versions of HEBCan to use in co-immunoprecipitation experiments with tagged forms of HEBAlt that had already been generated by another member of the lab (Figure 7A, B). The myc-tagged and HA-tagged constructs were co-transfected into 293T cells using Lipofectamine 2000. Twenty-four hours after the transfection, the cells were harvested using Pierce lysis buffer. Anti-myc antibody conjugated to agarose beads was incubated with the lysate overnight at 4°C in order to precipitate the myc-tagged proteins as well as any other proteins that were bound to them. The samples were run on an SDS-PAGE gel, transferred onto a PVDF membrane and then probed with an anti-HA or E2A-specific or Id protein-specific antibody to detect dimerized proteins.

HEBCan is able to form homodimers with itself and heterodimers with HEBAlt

Preliminary results from Ashton-Trotman Grant, a previous summer student at our lab, showed that HEBAlt is able to form homodimers in co-immunoprecipitation experiments. I set out to confirm this result and extend it to test dimerization with HEBCan.
Figure 7. Cloning tagged versions of HEB proteins to determine dimerization patterns. A. There is currently only an antibody that recognizes the common bHLH (basic helix-loop-helix) domain of HEB. Therefore, it is difficult to differentiate between HEBAlt and HEBCan proteins. In order to determine the interaction and dimerization patterns between different E proteins, tagged versions of the HEB proteins needed to be generated. B. HEBAlt and HEBCan were cloned into pCMV-HA and pCMV-Myc vectors. There is an antibody against the E2A protein. HEBCan was cloned into the MCS (multiple cloning site) of pCMV-HA and pCMV-Myc using the restriction enzymes Sal1 and Kpn1.
Figure 8. HEBCan and HEBAlt are able to form homodimers and heterodimers with each other. HEK293T cells were split at around 40% confluency one day before the transfection. The calcium phosphate method is used for the transient transfection. The cells are harvested using Pierce Lysis Buffer (with protease inhibitor) from Invitrogen 24 hours after transfection. After one hour of pre-clearing the lysate using Pierce Protein A/G agarose slurry at 4°C, co-immunoprecipitation is conducted by adding 40ul of c-myc monoclonal antibody-agarose beads (Clontech, clone 9E10) to pull-down the myc-tagged proteins and incubating at 4°C overnight. The samples are run on an SDS-PAGE Western gel and transferred onto a PVDF membrane. The membrane is probed with an anti-HA rabbit polyclonal Ab (from Takara Clontech) at a 1:100 dilution. The secondary antibody used is goat-anti-rabbit-HRP (1:1000). The blot is visualized using Clarity ECL kit (Biorad) and the Fusion Fx chemiluminescence and Fluorescence Imaging machine (Vilber Lourmat). The blot was exposed for 20 minutes. HEBCan is 85kDa and HEBAlt is 65kDa in size.
In Figure 8, the sample in the first lane contained the co-transfected pCMV-HEBAI-HA and pCMV-HEBAI-Myc. The lysate was immunoprecipitated using anti-myc agarose beads and then the blot was probed with anti-HA. The expected size of HEBAlt is around 65kDa. If the myc-tagged HEBAlt was able to dimerize with the HA-tagged HEBAlt, we would expect a single band located around 65kDa. This is indeed apparent in the first lane, indicating that HEBAlt was able to form homodimers. The second lane with pCMV-HEBAI-Myc and pCMV-HA was the negative control. Therefore, I was able to confirm the ability of HEBAlt to form homodimers. The HEBCan protein is around 85kDa in size. In lane 3, myc-tagged HEBCan was co-transfected with HA-tagged HEBCan. Similarly to HEBAlt, the single band around 85kDa shows that HEBCan was able to form homodimers. Lane 4 was the negative control for HEBCan. Importantly, lane 5 shows that HEBCan and HEBAlt can form heterodimers with each other, because the anti-HA antibody detected the pCMV-HEBAI-HA that was immunoprecipitated along with the pCMV-HEBCan-Myc. The lower 50kDa band that is present in all the lanes is due to detection of heavy chains of the primary antibody by the secondary antibody.

*E2A (E47) is able to form heterodimers with HEBCan and HEBAlt*

It is already known that E47 homodimers are important for B cell development (4). I was interested in determining whether E2A is able to form complexes with HEB factors, and conducted co-immunoprecipitation experiments to address this question. In Figure 9, the sample in lane 1 (the lane after the ladder) was co-transfected with pCMV-HEBCan-myc and E2A. The lysate was incubated with anti-myc agarose beads and the blot was probed with anti-TCF3/E2A. The presence of the lower band slightly above 70kDa shows that E2A was able to heterodimerize with HEBCan. It is unclear what the top band in lane 1 is, but it was speculated to be due to
Figure 9. E2A is able to form heterodimers with HEBCan and HEBAIlt. HEK293T cells were split at around 40% confluency one day before the transfection. The cells are transiently transfected using Lipofectamine 2000 and are harvested using Pierce Lysis Buffer (with protease inhibitor) from Invitrogen 24 hours after transfection. After one hour of pre-clearing the lysate using Pierce Protein A/G agarose slurry at 4°C, co-immunoprecipitation is conducted by adding 40ul of c-myc monoclonal antibody-agarose beads (Clontech, clone 9E10) to pull-down the myc-tagged proteins and incubating at 4°C overnight. The samples are run on an SDS-PAGE Western gel and transferred onto a PVDF membrane. The membrane is probed with an anti-TCF3/E2A rabbit polyclonal antibody (Abcam, ab82847) at a 1:100 dilution. The secondary antibody used is goat-anti-rabbit-HRP (1:1000). The blot is visualized using Clarity ECL kit (Biorad) and the Fusion Fx machine. The blot was exposed for 27 seconds.
cross-reactivity of HEBCan with the anti-E2A antibody. Future experiments will be done to address this question by probing cells singly transfected with pCMV-HEBCan-Myc with the anti-E2A antibody. Lane 2 is the negative control, with the empty pCMV-Myc co-transfected with E2A. The third lane shows that E2A is also able to form heterodimers with HEBAlt. Therefore, I have shown for the first time that HEBAlt is able to form dimers with the three major E proteins expressed during the early stages of T cell development.

**E2A, HEBCan and HEBAlt do not synergize in their activation of the RORγt promoter**

After observing all the different binding patterns between E proteins, I was interested to find out whether they cooperate with each other synergistically or combinatorially to activate RORγt transcription using dual luciferase reporter assays. For the luciferase assay shown in **Figure 10**, I singly transfected the E protein expression plasmids along with the RORγt-pGL4 promoter-reporter construct and also doubly transfected different combinations of E protein heterodimers (HEBCan/HEBAlt, HEBCan/E2A, and HEBAlt/E2A) to see whether there would be any synergistic or combinatorial effects. However, by comparing the luciferase signal for samples 5-7, I did not observe increases in the luciferase activity when there was co-transfection of the E protein expression constructs. Therefore, although all of the E proteins were able to activate transcription, they did not act synergistically or combinatorially to activate the RORγt promoter to a greater extent in the context of 293T cells. Importantly, we also observed that HEBAlt did not interfere with activation by either HEBCan or E2A, indicating that in this context it does not act like an Id factor in spite of the lack of the AD1 activation domain.

**Structure-function analysis of the alternative domain of HEBAlt**

*Generation of HEBAlt truncation mutants*
Figure 10. E2A does not act synergistically or combinatorially with HEB factors to activate the RORγt promoter in the context of HEK293T cells. 600,000 HEK293T cells are plated in 6 well plates the day before transfection. On the day of transfection, the cells are at an 80% confluency and were transfected using Lipofectamine 2000 (Invitrogen). 500ng of the RORγt-pGL4.10 [luc2] 2kb promoter-reporter construct was transfected, along with 50ng of pRL-thymidine kinase (TK renilla) construct and 1ug of either the HEBCan, HEBAlt or E2A expression plasmid, or 750ng of combinations of any two of the plasmids together. The empty pCMV-HA vector was used as a negative control. The data is from two independent experiments. An unpaired parametric t-test was performed to check for significance. ** p≤0.01  *** p≤0.001  **** p≤0.0001
The function of the unique and highly conserved 23 amino acid alternative domain of HEBAlt has largely remained elusive. Since we have now established that HEBAlt is able to positively regulate the ROR\(\gamma\)t promoter in the context of 293T cells, we were interested in determining whether specific regions or residues of the Alt domain are necessary for this function. I therefore set out to generate different truncations of the alternative domain to see whether the deletions would affect the transactivation of ROR\(\gamma\)t. The alternative domain contains three naturally occurring methionine residues that divide the alternative domain into three roughly equal portions in terms of amino acid length. I decided to use these methionines to generate three different truncation mutants: HEBAlt-Met2 starts at the second methionine residue and is missing the first 1/3 of the alternative domain; HEBAlt-Met3 starts at the third methionine residue and is missing the first 2/3 of the alternative domain; HEBTr does not contain the alternative domain at all (Figure 11A). All three truncation mutants had previously been cloned into the MIGR1 (MSCV-IRES-GFP) retroviral backbone. My initial plan was to transflect these constructs into 293T cells along with the ROR\(\gamma\)t-pGL4 promoter-reporter construct and perform dual luciferase reporter assays to see whether HEBAlt was still able to activate the ROR\(\gamma\)t promoter despite lacking portions of the alternative domain. However, we were concerned that since the dual luciferase reporter assay is a luminescence-based assay, the GFP present in the MIGR1 vector might interfere with the measurement of the luciferase signal, and preliminary tests did show problems. Therefore, I decided to PCR amplify HEBAlt truncation mutant inserts out of the MIGR1 backbone using specifically designed primers and subclone them into the pcDNA3.3-TOPO expression vector instead (Figure 11B). This vector allows for the cloning of the PCR product of interest upstream of the full-length human cytomegalovirus (CMV)
Figure 11. Generation of HEBAlt truncation mutants by deleting portions of the alternative domain. A. HEBAlt differs from HEBCan only by a highly conserved 23 amino acid Alt (alternative) domain that replaces the AD1 (activation domain 1) of HEBCan. This alternative domain contains three naturally occurring methionine residues. The truncation mutants are generated by cleaving off a portion of the Alt at the location of the methionine residues to determine which portion is indispensable for the function of HEBAlt. HEBAlt-Met2 contains 2/3 of the Alt domain, HEBAlt-Met3 contains 1/3 of the Alt domain and HEBTr is lacking the entire Alt domain. These inserts are cloned into the MIGRI (MSCV-IRES-GFP) retroviral backbone. B. The pcDNA3.3 TOPO vector allows for the cloning of the PCR product of interest upstream of the full-length human cytomegalovirus (CMV) immediate-early promoter/enhancer. Specific PCR primers were designed for each of the HEBAlt truncation mutants and the PCR products are gel purified and subcloned into the pcDNA3.3 TOPO vector. NheI-HF and XmaI were used to determine whether the inserts are incorporated in the correct orientation.
immediate-early promoter/enhancer and allows for high level expression of the protein of interest in adherent mammalian tissue culture cells following transient transfection.

**HEB that lacks the entire Alt domain does not activate transcription**

With the three HEBAlt truncation mutants cloned into the pcDNA3.3-TOPO vector, I conducted dual luciferase reporter assays with these constructs. Using the empty pcDNA3.3-TOPO vector as a normalizing control, I observed that there was only about a 1.5-fold increase in luciferase signal, compared with the 2.5-fold increase that was seen when the pCMV-HEBAlt-HA vector was used (Figure 12). Even though both vectors are driven by the CMV promoter, it appears that the pcDNA3.3-TOPO vector induces weaker expression of the gene of interest compared with pCMV-HA. However, I did observe that for the HEBAlt truncation mutants, HEBTr yielded a statistically significant decrease compared to the HEBAlt WT near back to baseline. This suggests that at least some residues of the HEBAlt domain are indispensable for the transcriptional activation of RORγt.

Using the lysate from the dual luciferase assay shown in Figure 12, I did a Western blot to check for the level of HEBAlt protein expression in the 293T cells. The blot was probed with an anti-HEB antibody, and pCMV-HEBAlt-HA was used as a positive size control (Figure 13). It is evident from these results that there is much higher expression of the HEBAlt truncation mutants compared to the HEBAlt WT in the pcDNA3.3-TOPO vector, which could have led to an overestimation of the transactivation ability of the mutants relative to wild-type. Future experiments will be needed to further assess the relationships between structure, stability, expression levels, and activity of proteins with different segments of the Alt domain included.

**Investigating the phosphorylation of tyrosine residues in the Alt domain**

*Generation of HEBAlt point mutants*
Figure 12. **HEB that lacks the entire Alt domain does not activate transcription.** 600,000 HEK293T cells are plated in 6 well plates the day before transfection. On the day of transfection, the cells are at an 80% confluency and were transfected using Lipofectamine 2000 (Invitrogen). 500ng of the RORyt-pGL4.10 [luc2] 2kb promoter-reporter construct was transfected, along with 50ng of pRL-thymine kinase (TK renilla) construct and 1ug of either of the three different HEBAlt alternative domain truncation mutants (HEBAI-t-Met2, HEBAlt-Met3, HEBTr). The empty pcDNA3.3 TOPO vector was used as a negative control and the HEBAlt WT is used as a positive control. The data is from two independent experiments. An unpaired parametric t-test was performed to check for significance. n.s. p>0.05 *p<0.05 **p<0.01
Figure 13. Confirming the protein expression of HEB in dual luciferase reporter assay. Lysates from the dual luciferase reporter assay from Figure 17 were used in a Western to test for the level of protein expression of the HEB truncation constructs cloned into the pcDNA3.3 TOPO vector. pCMV-HEBAlt-HA was used as a positive size control and the lysate comes from a separate experiment. The samples are run on an SDS-PAGE Western gel and transferred onto a PVDF membrane. The membrane is probed with an anti-HEB rabbit polyclonal antibody (Santa Cruz Biotechnology, A-20, sc-357) at a 1:500 dilution. The secondary antibody used is goat-anti-rabbit-HRP (1:1000). The blot is visualized using Clarity ECL kit (Biorad) and the Fusion Fx machine. The blot was exposed for 5 seconds. The HEB band is around 70kDa.
Preliminary data from our lab has shown that there is possibility of tyrosine phosphorylation of HEBAlt in SCID.adh and HeLa cells (not shown). The alternative domain of HEBAlt contains several tyrosine residues that may be potential targets of phosphorylation. I decided to make three different HEBAlt point mutants by mutating certain tyrosine residues to phenylalanines (Figure 14). Phenylalanine was chosen because it is the most structurally similar to tyrosine and would therefore constitute the most conservative mutation. HEBAlt MYCA 1F and HEBAlt 1F both have one mutated tyrosine residue, while HEBAlt 3F has three mutated tyrosine residues. The point mutations were created by designing specific primers and using the Quickchange mutagenesis kit to do site directed mutagenesis (Stratagene). All three point mutants were generated in the MIY (MSCV-IRES-YFP) backbone. The HEBAlt point mutant constructs were transiently transfected into 293T cells and the cells were harvested 24 hours after the transfection. The lysates were immunoprecipitated using anti-HEB antibody (clone A20, which detects both HEBAlt and HEBCan) and the Western blot of the immunoprecipitated lysates was probed with an anti-phosphotyrosine antibody (clone 4G10). The experiment was attempted once, and further optimization needs to be done in order to address the question of how tyrosine phosphorylation of the alternative domain might be related to the function of HEBAlt.

**The interaction between Id factors and E proteins**

*Id2 is able to form heterodimers with HEBCan and HEBAlt*

Id factors belong to the class V bHLH (basic-helix-loop-helix) proteins and lack the basic DNA binding region. They still have an intact HLH domain that is able to interact with and bind E proteins and inhibit their activity by sequestering them away from the DNA. To assess whether Id2 could dimerize with HEBAlt or HEBCan, I proceeded to do an immunoprecipitation. pCMV-HEBCan-Myc and pCMV-HEBAlt-Myc were singly transfected into 293T cells using
**Figure 14. Generation of HEBAlt point mutants by mutating tyrosine residues to phenylalanines.** The alternative domain of HEBAlt contains 23 unique and highly conserved amino acids, including tyrosine residues. Preliminary results show that there is possibility for tyrosine phosphorylation of HEBAlt. Three different HEBAlt point mutants were generated by mutating certain tyrosine residues in the Alt domain to phenylalanines. Specific mutagenesis primers were designed and the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate the mutations. The HEBAlt mutants were clone into a MIY (MIGR1-IRES-YFP) backbone.
Figure 15. Id2 is able to form heterodimers with HEBCan and HEBAlt. The Myc tagged HEB constructs were transiently transfected into HEK293T cells using Lipofectamine 2000. The empty pCMV-Myc vector was used as a negative control. One day after the transfection, the cells are harvested using Pierce Lysis Buffer (with protease inhibitor) from Invitrogen. After one hour of preclearing the lysate using Pierce Protein A/G agarose slurry at 4°C, co-immunoprecipitation is conducted by adding 40ul of e-myc monoclonal antibody-agarose beads (Clontech, clone 9E10) to pull-down the myc-tagged proteins and incubating at 4°C overnight. The samples are run on an SDS-PAGE Western gel and transferred onto a PVDF membrane. The membrane is probed with an anti-Id2 rabbit polyclonal antibody (Santa Cruz Biotechnology, C-20, sc-489) at a 1:200 dilution. The secondary antibody used is goat-anti-rabbit-HRP (1:1000). The blot is visualized using Clarity ECL kit (Biorad) and the Fusion Fx machine. The blot was exposed for 40 seconds.
Lipofectamine 2000 (Figure 15). The empty pCMV-Myc vector was used as a negative control. 24 hours after the transfection, the cells were harvested and the lysate was incubated overnight with anti-myc agarose beads to pull down the myc-tagged proteins. The samples were run on a SDS-PAGE gel and the blot was probed with an anti-Id2 antibody. The Id2 protein is around 15kDa. Therefore, the lowest two bands around 15kDa for lanes 2 and 3 show that HEBCan and HEBAlt are both able to heterodimerize with Id2. I proceeded to use the same approach to detect Id3 binding, but encountered issues with the anti-Id3 antibody (C-20, Santa Cruz Biotechnology, Dallas, Texas) after several attempts. Further optimization of this assay will need to be done in order to determine whether Id3 can heterodimerize with both HEB factors.
Chapter 4: Discussion and Future Directions
DISCUSSION

It is evident that E proteins play important roles in lymphocyte development, including regulating the rearrangement and expression of the immunoglobulin and TCR loci, antagonizing alternative fates, controlling survival and cell cycle progression, mediating lymphocyte selection and modulating chromatin remodeling. Our lab, in particular, has been focused on the study of HEB factors in T cell lymphopoiesis as well as investigating how these E proteins interact with each other in different gene regulatory networks. The establishment of a working system for the measurement of E protein expression and dimerization was an important first step toward a better understanding of E protein function. I found that the co-transfection of E protein expression plasmids along with a promoter-reporter construct in 293T cells and using a dual luciferase reporter assay as the experimental readout works adequately well. My studies showed that HEBAlt can transactivate transcription and that the absence of the alternative domain disrupts this function. Tagged HEB constructs were generated to facilitate the study of E protein dimerization, and I was able to show that all possible HEB and E2A dimers can be formed in vivo, in cultured cells. This system will allow the further study of the interactions between E proteins and other transcription factors involved in lineage choices.

Since it is known that E proteins function in a highly context-dependent way, the testing of different cell lines in optimized conditions was necessary to determine the best context in which to study E proteins. The non-adherent mouse thymoma EL4 cell line was first used, because that was the cell line used by Zhang et al (30) for their experiments showing the positive regulation of the RORγt promoter by E proteins. However, this cell line was very hard to transfect and gave variable results. The MNK-3 cell line was the next proposed candidate, because it has been shown to express RORγt and is a good in vitro system for the study of ILC3/LTi cells, which
express endogenous RORγt. However, based on the results shown by Xi et al (29), we thought that the MNK-3 cell line might not be ideal to use because of the high expression of Id2, which could suppress the ability of E proteins to induce strong levels of activation of the RORγt promoter. It is interesting to note that ILCs are highly Id2-dependent, but RORγt is still highly expressed in ILC3 cells despite the high expression of Id2. This suggests that the activation of RORγt may be context-dependent and driven by different promoters in different cell lines.

Since E proteins function as obligate dimers, it was of interest to us to define the different E protein binding partners and types of dimers that can be formed during T cell development. We know that AD1 and AD2 cooperatively recruit co-activators (p300/CBP, GCN5) and a co-repressor (ETO-2) in order to dynamically switch transcription between active and repressive states (8). The LDFS motif present in AD1 has been shown to mediate the interaction with the GCN5 co-activator, and the DES (downstream ETO-interacting sequence) downstream of AD1 is able to recruit co-repressors from the ETO (eight twenty-one) family proteins (8). AD1 also contains a conserved PCET (p300/CBP and ETO target) motif that is able to interact with either the p300/CBP co-activators or the ETO family co-repressors in a mutually exclusive manner. Since HEBAlt lacks the AD1 domain, it will be of interest to investigate whether it has the ability to recruit these co-factors as well and what roles they may play in its function. Furthermore, it has been shown that the nuclear receptor co-repressor (NCoR) is able to mediate ligand-dependent repression of the class II HLH protein MyoD and disrupt myogenic differentiation (36). It was discovered that the N-terminal repression domain (RD1) of NCoR is able to directly interact with the bHLH region of MyoD, which actually encodes a minimal repressor domain (36). Further experiments can be done in order to see whether HEBAlt is able to recruit NCoR and the effects that will have on early T cell development.
Based on my thesis, we can conclude that there are at least 7 different dimer combinations for E proteins: HEBCan homodimers, HEBAlt homodimers, HEBAlt/HEBCan heterodimers, HEBCan/E2A heterodimers, HEBAlt/E2A heterodimers, HEBCan/Id2 heterodimers and HEBAlt/Id2 heterodimers (Figure 16). E proteins are known to function in a highly dose-dependent way. Zhuang et al. (37) have shown that a combined dosage of all three E proteins (E2A, HEB and E2-2) is required for B cell development. It has been shown that E proteins can sometimes play redundant roles and compensate for the loss of each other. Interestingly, two bands were observed when pCMV-HEBCan-myc was co-transfected with E2A and probed anti-E2A antibody. This extra band could be due to cross-reactivity of HEBCan with the anti-E2A antibody. One way to address this question would be to try probing cells that are singly transfected with pCMV-HEBCan-Myc with the anti-E2A antibody to see if the top band is still visible.

Remarkably, not much is known about the relative affinities of E2A for the HEB proteins. In my experiment, I noticed that the E2A band was weaker for the co-transfection of pCMV-HEBAIAlt-myc with E2A compared with pCMV-HEBCan-myc. This could suggest that HEBCan/E2A heterodimers are favored over HEBAlt/E2A heterodimers because they are more stable, or it could also mean that HEBAlt might recruit co-repressors that reducing the interaction and binding with E2A. I also showed that E2A does not act synergistically or combinatorially with HEB factors to activate the RORγt promoter in the context of 293T cells. In other words, it appears that E2A does not cooperate with HEB factors to increase the activation of the RORγt promoter. A previous member of our lab has shown that HEB factors can actually inhibit the function of E2A in the context of epithelial HeLa cells (25). The co-transfection of either HEBCan or HEBAlt with E2A with an 8X E-box reporter construct led to a decrease in
Figure 16. All combination of E proteins dimers. Based on the results from my thesis, I have shown that there are seven different combinations of E proteins dimers: HEBAI_I homodimers, HEBCan homodimers, HEBCan/HEBAI_I heterodimers, HEBCan/E2A heterodimers, HEBAI_I/E2A heterodimers, HEBCan/Id2 heterodimers and HEBAI_I/Id2 heterodimers.
luciferase signal in DLR assays (25). It was also shown that HEB factors might inhibit early B cell development by disrupting E2A homodimer function. However, my results showed that the presence of HEB factors with E2A did not cause an inhibition of the transactivation of the RORγt promoter, so this is not a universal property of HEBAlt in all contexts.

I used Western blot analysis to check for the protein expression of HEBAlt and the HEBAlt truncation mutants and compare the level of expression induced by two different vectors: pCMV-HA and pcDNA3.3-TOPO. Interestingly, even though both vectors are driven by the CMV enhancer and immediate-early promoter elements, the pCMV-HA vector produced stronger protein expression than the pcDNA3.3-TOPO vector. Furthermore, we also see higher protein expression for all three HEBAlt truncation mutants (Met2, Met3, Tr) compared with the HEBAlt WT in the pcDNA3.3-TOPO vector. This could suggest that deletion of certain portions of the alternative domain of HEBAlt allows for increased protein stability. This discrepancy might also be due to the differences in cell lysis method, as the lysates are made by using the Passive Lysis Buffer (PLB) that comes from the DLR assay kit, instead of the usual Pierce lysis buffer that is used to make lysates for running on SDS-PAGE gels.

Most of the research conducted so far has been on E2A and HEBCan, but not much is known yet about the function of HEBAlt. One of the main goals of my thesis was to discover the function of the alternative domain of HEBAlt through structure-function analysis. We know that apart from the alternative domain, the rest of the structure of HEBAlt is exactly the same as HEBCan. We are interested to find out how the replacement of AD1 with the alternative domain differentiates the function of HEBAlt and alters its interaction with other transcription factors. Preliminary data shows that there is presence of tyrosine phosphorylation in the alternative domain of HEBAlt in SCID.adh and HeLa cell lines. We are interested in finding other whether
the phosphorylation of specific tyrosine residues are indispensable for the function of HEBAlt and other upstream or downstream factors that may interact with HEBAlt. A precedent for this would be the JAK (Janus kinase)-STAT (signal transducer and activator of transcription) signaling pathway, where JAK activation occurs after ligand-mediated receptor multimerization, followed by trans-phosphorylation, which allows for the further phosphorylation of other downstream targets such as STATs (38). The cascade of phosphorylation events allows transcription factors to be translocated to the nucleus and promote of transcription of target genes. It has been postulated that the alternative domain may allow HEBAlt to function as a transcriptional repressor in certain contexts, but further research is necessary to validate this hypothesis.

**FUTURE DIRECTIONS**

Since we already know that Id2 is able to form heterodimers with HEBCan and HEBAlt, the next step would be to find out whether Id3 is able to do the same. Several preliminary experiments were conducted with an anti-Id3 antibody (C-20, Santa Cruz Biotechnology), but experimental conditions need to be optimized in order to answer this question. Furthermore, in order to find out whether the alternative domain of HEBAlt is tyrosine phosphorylated, the conditions for the immunoprecipitation experiments involving the HEBAlt point mutants will need to be optimized as well. Once the HEBAlt point mutant constructs are singly transfected into 293T cells, the lysates will be incubated with the anti-HEB antibody, run on an SDS-PAGE gel, and the blot will be probed with the 4G10 anti-phosphotyrosine primary antibody. After we determine whether the alternative domain of HEBAlt can be phosphorylated, analysis of the point mutants will help us to narrow down on which tyrosine residues are important for its function. In addition, there are alternative targets of phosphorylation that can be investigated, as
there are eight tyrosine residues in the shared portion of HEBAlt and HEBCan and five tyrosines in the HEBCan-specific N-terminus.

Since context is very important for the function of E proteins, another thing to consider is to repeat the experiments in different cells lines, such as SCID.adh, which is a murine thymic lymphoma cell line that serves as a good in vitro model for the study of DN3 cells and early T cell development. It was speculated that the function of HEBAlt may rely on the recruitment of other co-activators or co-repressors. Furthermore, other E protein-responsive promoters such as the Notch1 (39) and pTα promoter (22) could be tested to see if they would yield higher signals in dual luciferase reporter assays.

**CONCLUSION**

In summary, it is evident that E proteins are major players in regulating lymphopoiesis. The expression levels and timing of E proteins need to be tightly regulated and fine-tuned, as breakdown of components of the gene regulatory network that they are involved in will result in serious defects and aberrant function in the immune system. Future research will focus on the interactions between HEBAlt and other factors in regulating early T cell development. A better understanding of E protein function will be crucial in elucidating the mechanisms by which immune abnormalities result and yield clinical applications.
REFERENCES

13. J. Wojciechowski, A. Lai, M. Kondo, Y. Zhuang, E2A and HEB are required to block thymocyte proliferation prior to pre-TCR expression. *J Immunol* **178**, 5717-5726 (2007); published online EpubMay
29. H. Xi, R. Schwartz, I. Engel, C. Murre, G. J. Kersh, Interplay between RORgammat, Egr3, and E proteins controls proliferation in response to pre-TCR signals. *Immunity* **24**, 813-826 (2006); published online EpubJun
Tables
Table 1. Sequences of primers used in these studies. Sequences are all shown 5’ to 3’.

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<th>Primer name</th>
<th>Primer Sequence</th>
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<tr>
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Table 2. Summary of Amaxa nucleofection trials using pMAX-GFP. Yellow indicates the programs that were used in the dual luciferase reporter assay shown in Figure 5.

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