Investigating the canonical Wnt signaling pathway in hematopoiesis: revisiting the role of Tcf-1 in early lymphoid development

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ABSTRACT

In hematopoiesis, the transcriptional networks governing lineage choice from pluripotent progenitors to mature lymphoid cells remain poorly understood. Although some key factors associated with the specification and commitment of T cells have been described, the functional hierarchy and the roles of key regulators in orchestrating these developmental programs remain unclear. While a loss-of-function model established the importance of T-cell factor 1 (Tcf-1), a T-cell specific mediator of Wnt signaling, in committed T-cell progenitors, I wanted to address how early in lymphoid development Tcf-1 is required. My work has aided in assigning functional significance to Tcf-1 as a gatekeeper of T-cell fate. I show that Tcf-1 is directly activated by Notch signals. Activation of Notch signaling in uncommitted precursors by the thymic stroma is known to initiate the T-cell differentiation program, and I show that Tcf-1 is required at the earliest phase of T-cell determination for progression beyond the early thymic progenitor (ETP) stage. This requirement is cell-intrinsic and Wnt-independent. The global expression profile of Tcf-1 deficient progenitors indicates that basic processes of DNA metabolism are downregulated in its absence, and the blocked T-cell progenitors die by apoptosis. While Tcf-1 is dispensable for the development of bone marrow (BM) progenitors and their migration through the blood, I present evidence for a novel role for Tcf-1 in the early development of natural killer (NK) cells in the BM. Together my data indicate a role for Tcf-1 earlier than previously described and demonstrate multiple roles for Tcf-1 in the roadmap of lymphoid development.

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CHAPTER I:

INTRODUCTION

1.1 The Immune System

The different cells that comprise the immune system allow for the recognition of various pathogens and an appropriate immune response. One arm of the immune system, termed innate, achieves this through conserved, hereditary receptors. Cells of the innate immune system include macrophages, dendritic cells (DC), and natural killer (NK) cells. The second arm, termed adaptive, can recognize pathogen through somatically rearranged receptors. T cells and B cells are part of the adaptive immune system, and are named for the thymus and bursa of Fabricius (in birds), respectively, for the organs in which they were first isolated and described. The proper development of the immune system is critical for recognition of pathogenic agents and is thus important for protection against disease.

Fascinatingly, all cells of the immune system can originate from a single, pluripotent cell, known as the hematopoietic stem cell (HSC). The HSC progresses through a series of stages with increasing lineage restriction, or decreasing multipotency, to yield distinct populations of immune cells with diverse phenotypic and functional properties. Distinct signatures of surface antigens, genes expressed, and transcription factor activity can identify the intermediate stages through which the cell differentiates. These transcription factors often have recurring roles in hematopoiesis, or the development of the immune system, but they may also be required to promote differentiation, survival, proliferation, or maintenance of a specific subset of immune cells. The

regulatory networks that instruct different cell fates are being intensely researched.

T cells and NK cells mediate immunity from the adaptive and innate components of the immune system, respectively. While having distinct functions in the immune response, they are closely related in their ontogeny, and some common transcription factors are critical in the regulation of both. Herein is described the development of T and NK cells from the HSC and an introduction to the transcriptional networks that govern these lineages

1.2 Lymphopoiesis

1.2.1 Hematopoietic stem cells and adult hematopoiesis

During mammalian embryogenesis, HSCs originate in the dorsal aorta, aorta-gonad-mesonephros (AGM) region, and the umbilical and vitelline arteries before they appear in the placenta and the yolk sac (YS). HSCs can then enter the circulation to colonize the fetal liver (FL). The liver does not generate hematopoietic cells *de novo* but is instead colonized by hematopoietic cells made in other tissues, and this additive contribution of HSCs to the liver may explain the large number of progenitors found there. HSCs expand in the FL, which is the major site of hematopoiesis during embryonic development, seed the spleen and thymus, and finally engraft the bone marrow (BM), which is maintained as the source of HSCs in the adult (Pina and Enver, 2007).

Interestingly, HSCs from FL and from the adult BM have been described as having different potentialities. In addition to differential requirements for

various factors in HSC maintenance, FL may be able to give rise to certain innate-like lymphocytes, which adult BM can no longer produce (Pina and Enver, 2007). For example, in the development of T cells, FL and adult HSCs show different *Vgamma* gene usage (Elliott et al., 1988). However, the best described difference in fetal and adult HSC potentiality has been in the description of $CD5^+$ B-1 B cell, which are of fetal origin (Hardy and Hayakawa, 1986; Herzenberg et al., 1986). Despite some documented differences, FL and adult BM HSCs have often been used interchangeably experimentally.

In the adult, HSCs are found in bone marrow, where all blood cells originate. The earliest precursor in the bone marrow, termed the long-term (LT)-HSC (**Figure 1.1**), has the potential to differentiate into all cell types of hematopoietic origin and is capable of self-renewal. The surface phenotype of the LT-HSC is described as being negative for all so-called lineage* markers that define mature blood cells (Lin^-) and positive for stem cell antigen-1 (Sca-1), and CD117 (c-kit). (*For example, lineage markers commonly include CD3 for T cells, CD19 for B cells, Gr1 for granulocytes, and Ter119 for erythrocytes.) (Adolfsson et al., 2001; Christensen and Weissman, 2001). Progenitors with this phenotype are referred to more generally as LSKs ($Lin^-Sca\text{-}1^+c\text{-}kit^+$).

Lymphoid priming, or the early activation of loci associated with the lymphoid gene program from an uncommitted progenitor, occurs as early as the HSC. Ikaros, encoded by the gene *Ikzf1*, has been shown to be required for the induction of lymphoid “signature” transcripts, including *Dntt*, *Satb1*, *Sox4*, *Foxp1*, *Flt3*, and *Notch1*, within these early progenitors (Ng et al., 2009; Yoshida et al.,

2006). Ikaros is a transcription factor containing an N-terminal zinc finger DNA-binding domain and C-terminal activation and zinc finger dimerization domains. Deletion of the last exon of *Ikzf1* encoding the activation and dimerization domains results in an Ikaros-null mutation. Ikaros expression in the hematopoietic system is broad, and Ikaros-null mice carry defects in T-, B-, NK, and dendritic cells (Georgopoulos et al., 1992; Nichogiannopoulou et al., 1999; Wang et al., 1996). In addition, Ikaros represses genes associated with self-renewal and multipotency in progenitors downstream of the HSC (Ng et al., 2009; Yoshida et al., 2006).

The LT-HSC is also marked by expression of the transcription factor, *Gata3* which has been described to have important roles in the maintenance of these cells (Ku et al., 2012). Gata3 is a member of the Gata zinc-finger transcription factors, of which three (Gata1, Gata2, and Gata3) are expressed in hematopoietic lineages. They are characterized by binding to the WGATAR DNA consensus motif (where W= A/T and R=A/G) (Hosoya et al., 2010). While Gata3 is first expressed in the LT-HSC, it also has critical roles in early thymic development (described in a later section).

Upregulation of the adhesion molecule CD34 on the surface of HSC marks the generation of the short-term (ST)-HSC. These cells also have the capacity to differentiate into all hematopoietic cell types, but are no longer able to self-renew indefinitely (Yang et al., 2005). Expression of PU.1, encoded by the gene *Sfpi1*, begins at the ST-HSC (Akashi, 2007; Iwasaki et al., 2005). PU.1 is a member of the Ets family of winged helix-turn-helix transcription factors

characterized by homology to the avian leukemia v-ets sequence. While indispensable for macrophage and B-cell development, PU.1 has known functions in various hematopoietic compartments (Scott et al., 1994). More specifically, PU.1 can affect development in lymphoid cells by direct control of the *Il7ra* gene, from which transcript can be detected at early stages in hematopoietic development (DeKoter et al., 2002).

Upregulation of the cytokine receptor fms-like tyrosine 3 (Flt3) correlates with the rise of the multipotent progenitor (MPP), which retains myeloid, granulocyte, and lymphoid but not megakaryocyte/erythrocyte potential (MegE) (Adolfsson et al., 2005). PU.1 is repressed at the MPP and later stages in BM hematopoiesis by growth factor independence (Gfi)1, which has additional important functions in thymic development (described in a later section) (Spooner et al., 2009). Gfi1 contains six C-terminal zinc-finger binding domains and a characteristic “SNAG” domain, which comprises the first 20 N-terminal residues and mediates repressor function (Grimes et al., 1996a; Zweidler-Mckay et al., 1996). Gfi1 is capable of binding a conserved motif in the promoter and the upstream regulatory element (URE) of the *Sfpi1* locus. Moreover, these Gfi1 binding sites overlapped with, or were in close proximity to, PU.1 binding sites thought to be important for an autoregulatory feedback loop. In a cell line expressing a 4-hydroxytamoxifen (OHT) inducible PU.1 protein (PUER), increased PU.1 binding to these autoregulatory sites lead to a loss of Gfi1 binding, suggesting that Gfi1 could be competitively displaced by PU.1 for binding to these sites (Spooner et al., 2009).

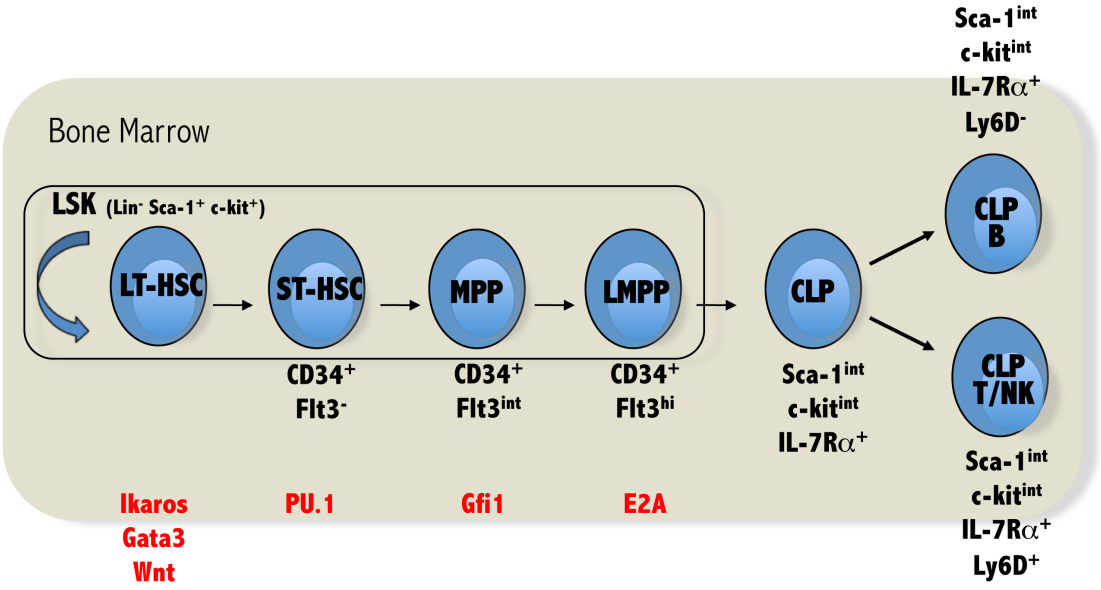

Figure 1.1: **A lymphoid-centric schematic of BM hematopoiesis.** LT-HSC can give rise to all cells of the immune system and has the capacity for self-renewal. Lymphoid development progresses through a series of intermediate progenitors with increasing lineage restriction. These stages are identified by surface markers for flow cytometric analyses (black). Several transcription factors or signaling pathways (red), which are activated at the stages indicated, have an important influence on lymphopoiesis.

Two pathways diverge from the MPP: one giving rise to the common myeloid progenitor (CMP) and the other giving rise to the $Flt3^{hi}$ lymphoid-primed multipotent progenitor (LMPP), which has the potential to develop into B, T, and NK cells (Adolfsson et al., 2005; Kondo et al., 1997). Similar to the role of Ikaros in the HSC, E2A is important for lymphoid priming at the LMPP, as evidenced by failure to express the lymphoid-specific genes *Notch1*, *Ccr9*, *Dntt*, and *Il7ra* in E2A-deficient mice (Dias et al., 2008). E2A, encoded by the gene *Tcf3*, is a member of the E protein family. These proteins bind to the E-box DNA motif and are characterized by a helix-loop-helix (HLH) dimerization domain, allowing them to form homo- or heterodimers with each other or other HLH proteins. These include a family of repressors that do not have the ability to bind DNA, the inhibitor of DNA-binding (Id) proteins. The requirement for E2A at the LMPP stage is dose-dependent as mice heterozygous for E2A deficiency exhibited reduced numbers of LMPPs, which were further reduced in homozygous mice (Dias et al., 2008).

LMPPs give rise to the common lymphoid progenitor (CLP). CLPs can be distinguished phenotypically from LSKs in that they express intermediate levels of Sca-1 and c-kit and show surface upregulation of the α-chain of the interleukin-7 receptor (IL-7Rα, CD127) (Karsunky et al., 2008; Kondo et al., 1997). Signaling through IL-7 is critically important for the proliferation and differentiation of lymphocytes; deficiency in IL-7 or components of its receptor results in developmental blocks in B and T cells (Peschon et al., 1994). CLPs are composed of precursors with either B-cell potential or with T/NK-cell potential,

the latter of which can be differentiated by surfaced expression of Ly6D (Inlay et al., 2009). Another CLP, termed CLP-2, has been identified as being CD45R $(B220)^+$ and can also yield T-cell progeny (Martin et al., 2003).

1.2.2 Blood circulating T-cell progenitors

T-cell identity is endorsed exclusively by the specialized environment of the thymus. BM precursors must therefore migrate through the blood stream and colonize the thymus. The major contributing cell population(s) that emigrate(s) from the bone marrow to seed the thymus are still unknown because of the paucity of early T cell progenitor populations and an inability to readily detect such cells circulating in blood. Moreover, none of the later lymphoid progenitors in the bone marrow possess a surface phenotype that quite resembles the early thymic progenitors (ETPs) (IL-7Rα^+c-kit$^+$). To date, three hematopoietic progenitors that differ in their surface phenotypes but are derived from blood have been shown to give rise to T cells when cultured in vitro. The first of these to be identified is the blood LSK (Lin$^-$Sca-1$^+$c-kit$^+$) (Schwarz and Bhandoola, 2004). The other two are the blood CLP (Lin$^-$Flt3$^+$IL-7Rα^+) and the circulating thymic progenitor (CTP). The latter population is also Lin$^-$Flt3$^+$IL-7Rα^+, but was originally identified using transgenic mice expressing human CD25 (HuCD25) under the control of the pTα promoter (Krueger and von Boehmer, 2007; Schwarz and Bhandoola, 2004; Umland et al., 2007). Expression of pTα is necessary for the development of αβ-T cells, and pTα is expressed by CD44$^+$CD25$^-$ DN1 cells.

While all the bone marrow precursors (from HSC to CLP-2) have some capacity to differentiate into T cells, they may do so with different efficiencies, and thus may have different contributions to the T-cell pool in a physiological context. Indeed, the primary determinant of which BM progenitors play a role in T-cell development may be the ability of a progenitor to traffic from the BM into the thymus. Chemokine receptors CCR9 and CCR7 have both been shown to be important for efficient migration of cells through the blood and entry into the thymus (Krueger et al., 2010; Schwarz et al., 2007; Zlotoff et al., 2010). Additionally, the tight regulation of chemokine receptor expression is necessary for intraorgan trafficking of progenitors through their respective thymic niches (Misslitz et al., 2004).

1.2.3 Early thymic progenitors, initiation of the T-cell fate, and maintenance of T-cellness

Blood progenitors entering the thymus are referred to as thymus seeding progenitors (TSP). Early thymic pro-T cells are referred to as being double-negative (DN) due to the absence of surface CD4 and CD8, and they progress through a series of developmental checkpoints through the immature single-positive (ISP) to the $CD4^+CD8^+$ double-positive (DP) and, finally, the $CD4^+$ or $CD8^+$ single-positive (SP) stage (**Figure 1.2**). The DN stages are designated as DN1 through DN4, and they are characterized by differential expression of the surface antigens CD44 and CD25 (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; and DN4, $CD44^-CD25^-$). The DN1 is a heterogeneous cell population, which can be further subdivided into DN1a through DN1e based on

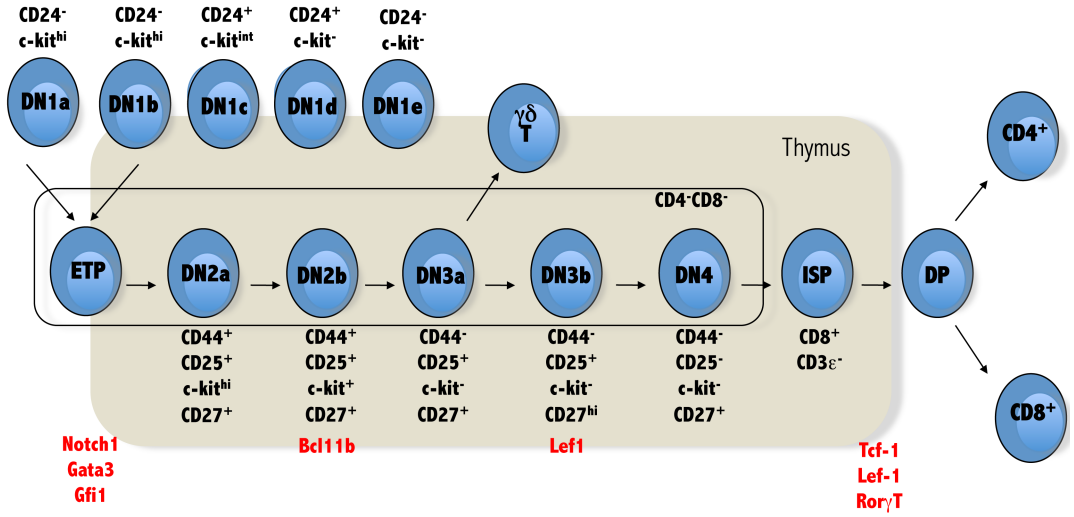

Figure 1.2: **A schematic of thymic development.** T cells are unlike other cells in the immune system in their requirement for a second site for completion of differentiation. The stages of thymic development are identified by surface markers for flow cytometric analyses (black). Several transcription factors or signaling pathways (red), which are activated at the stages indicated, have an important influence on T-cell development.

surface expression of c-kit and CD24 (heat stable antigen, HSA) (DN1a, c-kit^{+}CD24^{-}; DN1b, c-kit^{+}CD24^{lo}; DN1c, c-kit^{+}CD24^{+}; DN1d, c-kit^{-}CD24^{+}; and DN1e, c-kit^{-}CD24^{-}) (Porritt et al., 2004). Only the highest c-kit expressors (i.e. DN1a and DN1b) are considered true T-cell progenitors in that they give rise to T cells most efficiently.

The c-kit^{hi} DN1 cells are also referred to as the early thymic progenitor (ETP) and have been reported to retain the ability to give rise to B cells, DCs, NK cells, and macrophages (Allman et al., 2003; Bell and Bhandoola, 2008; Luc et al., 2012; Wada et al., 2008). However, evidence using an IL-7Rα Rosa-YFP reporter suggests that all true T-cell progenitors have expressed the IL-7Rα before entry into the thymus. Additionally, these studies provide evidence that thymocytes with T-cell potential and thymocytes with macrophage potential derive from separate progenitor origins (Schlenner et al., 2010). The ETP gene profile is described as most closely resembling a BM IL-7Rα^{+} LMPP, which differs from the CLP in that it expresses higher levels of c-kit (Luc et al., 2012).

The earliest and most critical determinant of T-cell fate in the thymus occurs at the ETP by signaling through Notch1, which specifies the T-cell lineage. Notch is an evolutionarily conserved receptor implicated in various developmental systems. In mammals there are four Notch receptors (Notch1-Notch4) and five ligands (Jagged1, J1; Jagged2, J2; Delta-like 1, Dll1; Delta-like 3, Dll3; and Delta-like 4, Dll4). Upon ligation of the extracellular domain of the single-pass Notch receptor, a proteolytic cleavage cascade is initiated resulting in the release of the cytoplasmic domain (intracellular Notch, ICN) by γ-secretase.

ICN can then translocate to the nucleus where it binds CSL, (also called recombination binding protein at Jκ, RBP-j in mice) to activate transcription of Notch target genes (Radtke et al., 2010) (see **Figure 1.3**).

Different Notch receptors have been implicated in various stages of lymphopoiesis. However, the best-characterized role for Notch in the development of the immune system has been in the differentiation of T cells in the thymus. Human *Notch1* was first identified as being contained within a locus involved in a translocation from a case of T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). This gene was highly homologous to the *Drosophila* Notch, which had already been linked with cell fate and spatial patterning.

Notch1 is critical for the specification of the T-cell fate by upregulating T-cell specific genes while simultaneously repressing B-cell fate. Genetic ablation of Notch1 or overexpression of Notch1 antagonists results in early arrest of T-cell development at the DN1 stage and enhanced thymic B-cell development, indicating that Notch signaling is sufficient to drive the T-cell fate (Feyerabend et al., 2009; Izon et al., 2002; Koch et al., 2001; Maillard et al., 2004; Radtke et al., 1999; Wilson et al., 2001). Reciprocal gain-of-function experiments have demonstrated that expression of ICN or Dll4 in BM progenitors results in the ectopic development of T cells in the BM and a block in B-cell development (Dorsch et al., 2002; Yan et al., 2001). Additionally, expression of the Notch1 targets and transcriptional repressors Hes1 and Hes5 in the BM also results in impaired B cell development, suggesting that Notch1 is able to suppress the B cell fate through induction of Hes (Kawamata et al., 2002).

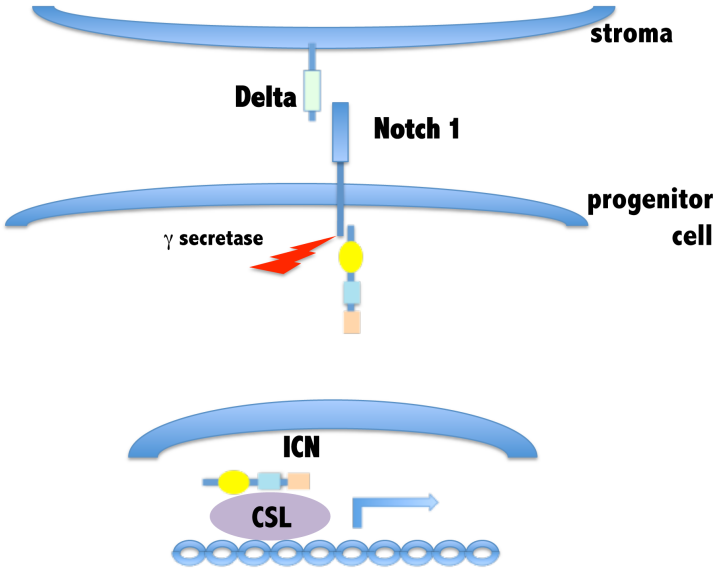

Figure 1.3: **A schematic of Notch signaling.** Binding of Notch receptors to their ligands, such as Delta, triggers a proteolytic cleavage cascade resulting in the release of an activated intracellular Notch domain, which translocates to the nucleus to bind CSL and activates transcription.

Ligation of Notch by its different ligands may result in different biological functions. While all Notch ligands are expressed on thymic stroma, only J1, J2, and Dll1 are expressed in the BM (Radtke et al., 2004). The ability of Notch to bind Jagged ligands is modulated in part by the glycosyltransferase, Fringe. The glycosylation of Notch by Fringe restricts its binding to Delta ligands only, and inhibits its binding to Jagged (Haltiwanger and Stanley, 2002). The stable transfection of the positively regulating Notch ligand Dll1 in the OP9 stromal cell line (OP9-DL1) has allowed for *in vitro* study of T-cell development (Schmitt and Zuniga-Pflucker, 2002).

While Notch1 signaling at the ETP is important for the specification of T-cells by upregulating T-cell-specific genes, other factors, which may also be regulated by Notch1, are required for the maintenance of this lineage before a progenitor can truly become a T cell. Exposure to Notch1 signals upon entry into the thymus leads to the loss of B-cell potential, presumably through the action of Notch-1 target Hes. However, early DN2 cells retain the potential to give rise to other cell fates. Additional factors are required for the suppression of these alternative fates, cellular maintenance of T cells, and the continued expression of genes required to achieve T-cell identity. An analogous system for the specification and maintenance of lymphoid fate has been described for B cells, where E2A and early B cell factor (EBF) initiate the B-cell program, Pax5 represses alternative fates in progenitor-B cells, and recurrent signals through Pax5 and other factors are required for the terminal differentiation of mature B cells (Busslinger et al., 2000).

Additional factors are critical for the development and maintenance of ETP. Gata3, whose role in the LT-HSC has already been discussed in a previous section, is one these. Even when it was first cloned, Gata3 was speculated to play a role in lymphoid development due to its high expression in T cells (Yamamoto et al., 1990). Using semi-quantitative PCR of fetal thymus (FT), it was noted that Gata3 (and Tcf-1) expression showed marked upregulation after embryonal day 12 (E12), when the FT is first colonized by progenitors and its phenotype is exclusively $CD44^{+}CD25^{-}$, indicating that Gata-3 was likely involved in the earliest stages of T-cell development. Indeed, addition of antisense Gata3 oligonucleotides to fetal thymic organ cultures (FTOC) inhibited development of $Lin^{-}c\text{-}kit^{+}$ FT cells as was demonstrated by the reduced recovery of $Thy1^{+}$ cells (Hattori et al., 1996). More recently, BM reconstitution of congenic hosts and culture on OP9-DL1 stromal cells with Gata3-null (*Gata3*$^{LacZ/LacZ}$) progenitors as well as analysis of Gata3-hypomorphic (*Gata3*$^{g/g}$) embryos demonstrated that Gata3 deficiency results in an early block in T-cell development that is not simply due to apoptosis or a failure to proliferate (Hosoya et al., 2009). While ablation of Gata-3 has profound defects in T-cell differentiation starting at the ETP, its overexpression at the DN2 stage also leads to a developmental block, and potentially, diversion to the mast cell lineage (Taghon et al., 2007). *Gata3* can be expressed from two different promoters to yield *Gata3-1a* or *Gata3-1b* transcripts. The latter is the form predominantly expressed in thymocytes (Asnagli et al., 2002).

Another critical factor in ETP development is Gfi1. Ablation of Gfi-1 results in severe thymic defects. *Gfi1*$^{-/-}$ mice exhibit reduced thymic cellularity, and the c-kit^{+} DN1 and DN2 populations are particularly affected due to increased apoptosis and a failure to proliferate (Yucel et al., 2003). Of note, other studies have indicated a role for Gfi1 in the repression of the pro-apoptotic factor Bax (Grimes et al., 1996b). *Gfi1*$^{-/-}$ thymocytes were reported to have increased expression of the HLH proteins Id1 and Id2, the latter of which has been described to promote NK-cell fate (described in a later section, (Boos et al., 2007).

1.2.4 Thymic Development: DN2 to mature T cell

Commitment to the T lineage occurs at the DN2 stage. The early DN2a progenitors can be distinguished from the T-committed DN2b based on the expression of higher levels of c-kit on their surface. This earlier stage retains the potential for macrophages, dendritic cells (DC), NK cells and mast cells (Taghon et al., 2007). *Bcl11b* expression increases markedly at the DN2 stage. Bcl11b is a Krüppel-like C2H2 type zinc-finger transcription factor and one of two members of the Bcl11 family. *Bcl11b*$^{-/-}$ mice show blocked T-cell development and increased thymic NK development. Furthermore, Bcl11b is required for the expression of T-cell specific genes and repression of both the NK and stem cell signature, demonstrating the role of Bcl11b in commitment to the T lineage at the DN2 stage (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b).

The HLH protein HEBalt plays a role in progenitor (pro)-T-cell development particularly at the DN2 and the transition to the DN3 stage. HEBalt

is an isoform of HEB expressed from the *Tcf12* gene but contains a unique N-terminal (Alt) domain due to expression from an alternative promoter. HEBalt expression begins at the DN2, declines at the DN4, and is absent at the DP. Retroviral transduction of HEBalt into $HEB^{-/-}$ T-cell progenitors can restore differentiation to the DN3 stage (Braunstein and Anderson, 2011; Wang et al., 2006).

The fate decision between γδ- and αβ-T cells occurs at the DN3 stage. During the DN3 stage, the recombinase activating genes 1 and 2 (*Rag1* and *Rag2*) are upregulated. This is required for the rearrangement of the T cell receptor (TCR). V(D)J recombination, which is catalyzed by Rag1 and Rag2, allows for the somatic rearrangement of clonally diverse receptors on adaptive immune cells (B and T). The *Tcrb* and *Tcrd* loci in T cells are characterized by the presence of multiple variable (V), diversity (D), and joining (J) regions. During rearrangement an exon in the D cluster is joined to an exon in the J cluster, then finally the rearranged DJ unit is joined to an exon in the V cluster. In order for this recombination to occur, Rag proteins are required to introduce DNA breaks to allow for the joining of non-adjacent gene segments by non-homologous end joining (NHEJ). If both the *Tcrd* locus, which is interspersed with the *Tcra* locus, and the *Tcrg* locus are productively rearranged first, a γδ-TCR-bearing cell forms (von Boehmer and Fehling, 1997). The high mobility group (HMG)-box factor sex-determining-region (SRY) box (Sox)13 is uniquely expressed in γδ-T cells, and has been reported to drive γδ-T cell development. Interestingly, Tcf-1 was

reported to antagonize Sox13 to promote αβ-T-cell development (Melichar et al., 2007).

On the other hand, productive rearrangement of TCRβ and formation of a functional pre-TCR complex with the invariable pTα chain allows cells to progress through the DN3b stage (β-selection). Cells in the DN3b stage can be distinguished phenotypically from cells in the earlier DN3a stage based on a higher expression of the surface antigen CD27 and larger cell size due to the high level of proliferation that occurs after β-selection (Taghon et al., 2006). A plethora of transcription factors have been implicated in β-selection, including E2A, HEB, and Notch1, which have already been introduced elsewhere in this section.

Gata3, c-Myc, and multiple other transcription factors mediate the progression of β-selected T cells through the DN4 stage. Upregulation of surface CD8 in the absence of surface CD3 marks the ISP, and upregulation of surface CD4 follows, leading to the DP stage. Termination of IL-7R signaling and the upregulation of orphan nuclear receptor (ROR)γt, lymphoid enhancer factor (Lef-1), and Tcf-1 are important for the transition from ISP to DP. In lymphoid cells, (ROR)γt is the predominant isoform of the *Rorc* locus, which is so-named for its homology to the retinoic acid receptor (RAR). At the DP stage, *Rag1* and *Rag2* are again upregulated, this time to rearrange the TCRα locus. Disruption of *Rorc* results in a reduction of DP and, consequently, mature T cells (Kurebayashi et al., 2000; Sun et al., 2000). This may be a result of a failure to upregulate the pro-survival factor Bcl-xL (Kurebayashi et al., 2000). Additionally, *RORγt* can bind

a putative consensus motif in the TEA element upstream of the *Ja* cluster of the *Tcra* locus, although there has been no physiological demonstration of rearrangement control (Villey et al., 1999). Positive selection occurs at the DP stage to yield CD8 or CD4 SP cells. Selection and the generation of mature T cells occur by complicated mechanisms that are actively being studied but are still incompletely understood (McCaughtry and Hogquist, 2008).

Naïve $CD4^+$ and $CD8^+$ T cells emigrate from the thymus to circulate in the lymph and populate secondary lymphoid sites, where activation will induce further development into effector and memory cells. $CD4^+$ T cells are often referred to as T helper (Th) cells, which include an ever-growing variety of members distinguished by differential cytokine production and consequent immune response. These include Th1, Th2, the IL-17-producing Th17, T regulatory (Treg), and T follicular helper (Tfh) cells.

1.2.5 NK cell development from the CLP

NK cells are innate lymphocytes that were so-named for their ability to "naturally" kill tumor cells without prior sensitization (Kiessling et al., 1975). NK can directly recognize and kill virally infected, distressed, or cancerous cells via germline-encoded receptors (Lee et al., 2007; Smyth et al., 2002). Additionally, NK cells can modulate the immune response via their interactions with B cells and DCs (Abruzzo and Rowley, 1983; Blanca et al., 2001; Spaggiari et al., 2001; Vitale et al., 2005a; Vitale et al., 2005b).

Development of NK cells diverges from other lymphocytic lineages at the CLP. Signaling through IL-15 is indispensable for NK-cell development. The IL-

15 receptor is composed of three critical subunits: the unique IL-15Rα subunit, the β subunit shared with IL-2 (IL-2Rβ, CD122), and the common γ chain (CD132), which is shared by several other cytokines. Mice lacking IL-15 or any components of the receptor have severe defects in NK cell development (DiSanto et al., 1994; Suzuki et al., 1997). The earliest NK-committed progenitor downstream the $Ly6D^+$ CLP was recently described as expressing 2B4 (CD244) and NKG2D but lacking expression of IL-2Rβ. These progenitors were designated pre-NK progenitors (pre-NK) (Fathman et al., 2011). Upregulation of CD122 marks NK progenitors (NKPs, $Lin^- CD122^+NK1.1^-DX5^-$) (Rosmaraki et al., 2001) (**Figure 1.4**). Further differentiation of NKPs is associated with the acquisition of additional families of activating and inhibitory NK receptors. Expression of the activating receptor NKR-P1C, which is the antigen recognized by the NK1.1 antibody, is used to define the immature (i)NK cell population ($Lin^- CD122^+NK1.1^+DX5^-$). While still incapable of lysis or cytokine production, iNKs begin to express the CD94 and NKG2 family of molecules, and the highly polymorphic and polygenic Ly49 family of major histocompatibility (MHC) I binding receptors (Kim et al., 2002; Rosmaraki et al., 2001). Acquisition of effector properties is first detected in mature NK (mNK, $Lin^- CD122^+NK1.1^+DX5^-$) cells in the BM and is associated with expression of the integrin CD49b (recognized by the DX5 antibody). Upon stimulation, BM mNK cells are able to mediate significant cytolysis and cytokine production and phenotypically resemble the mature NK cells found in peripheral tissues (Kim et al., 2002).

Few factors have been described in the ontogeny of NK cells, and the

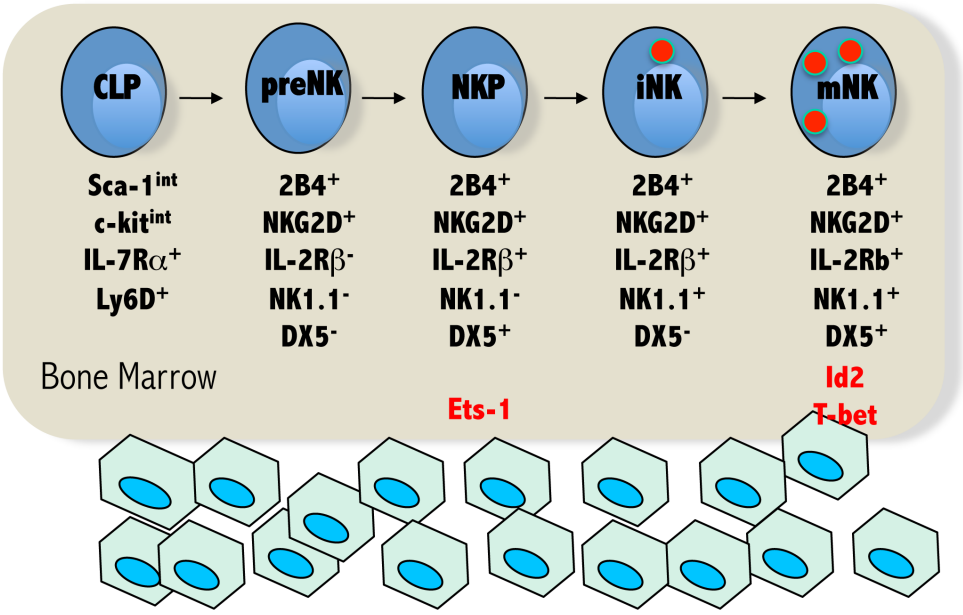

Figure 1.4: **A schematic of bone marrow natural killer cell development (BM NK).** NK cells diverge from other lymphoid lineages at the CLP. The stages of NK development are identified by defined surface markers for flow cytometric analyses (black). Little is known about the genes that regulate NK specification, but some of the transcription factors involved are indicated (red). The placement of these factors at specific stages of NK cell development have yet to be elucidated.

precise stages at which they function remain unclear. The Ets1 proto-oncogene is one of the first factors to be implicated in NK cell biology and is the founding member of the Ets family transcription factors, of which PU.1 is also a member. Using a *Rag2*$^{-/-}$ complementation system with ES cells carrying a targeted deletion in the Ets-1 DNA-binding domain first indicated a role for this factor in lymphoid development. These chimeric mice had reduced numbers of peripheral T and NK cells owing to impaired survival and proliferation (Bories et al., 1995; Muthusamy et al., 1995). A hypomorphic mouse carrying a deletion in exon 3 and part of exon 4 of *Ets1* was created for further analysis (*Ets1*$^{p/p}$, (Barton et al., 1998). CD3^{-}DX5^{+} BM NK cells are absent in *Ets1*$^{p/p}$, suggesting a role early in the development of this lineage. The mechanism for the involvement of Ets1 in NK differentiation may be by direct regulation of Id2 (Ramirez and Kee, unpublished).

Id2, which antagonizes the lymphoid factor E2A, has also been shown to play a role in NK-cell development. Enforced expression of Id2 in early human thymocytes blocked T-cell development while stimulating NK cell development in *in vitro* culture supplemented with IL-15, suggesting a synergy between Id2 and IL-15 to repress T-cell fate while promoting NK cell development (Schotte et al., 2010). A cell-intrinsic requirement for Id2 in NK cell differentiation was first demonstrated by the near complete lack of NK cells in the spleens of mice reconstituted with Id2-deficient bone marrow (Yokota et al., 1999). In adult mice, NKP and mNK cells but not CLPs express Id2. However, NKP and iNK cell numbers are unaffected in mice carrying a deletion in Id2, while mNK cells were

severely decreased. Combined deletion of Id2 and E2A rescued the BM phenotype, suggesting that Id2 is required to antagonize E-protein activity at the mNK stage; however *Id2*$^{-/-}$*E2A*$^{-/-}$ mice still showed reduced numbers of peripheral NK cells (Boos et al., 2007).

NK cell development also depends on T-box transcription factors. These factors form homo- or heterodimers to bind palindromic DNA sequences known as T-box binding elements. T-bet, encoded by the *Tbx21 gene*, was first identified in a screen of transcription factors that were differentially required in Th1 and Th2 cells (Szabo et al., 2000). Expression of T-bet and Eomesodermin (Eomes), encoded by the *Eomes* gene, overlaps in CD8^{+} T cells, Th1 T cells, and NK cells, suggesting redundancy in their control of target genes. Mice deficient in *Tbx21* display a cell-intrinsic reduction in the number of peripheral NK cells and a severe block in NKT cell development (Townsend et al., 2004). Deletion of an *Eomes* allele from *Tbx21* deficient mice (*Eomes*$^{+/-}$ *Tbx21*$^{-/-}$) resulted in a loss of nearly all IL-15 dependent lymphoid lineage cells, including CD8 memory T cells and mNK cells (Intlekofer et al., 2005). Loss of these lineages was found to be due to direct regulation of *Cd122* expression by Eomes and T-bet (Townsend et al., 2004). Critical roles for additional transcription factors in the development of NK cells, are under intense investigation.

1.3 Wnt signaling, Tcf-1 as its downstream effector, and roles in hematopoiesis

A variety of signaling pathways have been implicated in the differentiation, proliferation, and survival of developing T cells. One of these is the Wnt pathway, which is evolutionarily conserved in various species ranging from *Drosophila* to human. In the activation of the canonical pathway, members of the Wnt family bind a seven-transmembrane domain receptor of the Frizzled family. Humans and mice are known to express 19 Wnt molecules and 9 Frizzled receptors. These receptors are differentially expressed, depending on cell type, and appear to define responses to Wnt signal in various tissues and stages of development (Nusse, 2005).

The protein β-catenin was identified as the vertebrate homolog of the Drosophila segment polarity gene product Armadillo (McCrea et al., 1991), which was shown to interact with the *wingless* signaling pathway in *Drosophila* pattern formation (Peifer et al., 1991). Armadillo and other members of the ARM-repeat family are characterized by a 40-amino-acid-long motif of tandem repeats. These can fold together to form a superhelix that serves as an interface for interaction with many proteins. β-catenin was initially described as a 92-94 kDa protein associated with the cytoplasmic tail of cadherin cell adhesion molecules (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). In the absence of Wnt signals, β-catenin not found in adherens junctions is sequestered in the cytoplasm by the so-called “destruction complex,” which is composed of several proteins, including axin, adenomatous polyposis coli (APC), and glycogen

synthase kinase-3β (GSK-3β). Phosphorylation of β-catenin by GSK-3β targets it for ubiquitination and proteasomal degradation. The binding of Wnt to its receptor disrupts the "destruction complex," allowing for the accumulation of β-catenin and its translocation into the nucleus, where it competes for binding to the Tcf/Lef family of DNA binding factors. β-catenin recruits a host of other factors that mediate chromatin remodeling and transcriptional activation from Tcf/Lef-responsive promoters (Willert and Jones, 2006) (**Figure 1.5**).

Tcf-1 was linked to the evolutionarily conserved canonical Wnt signaling pathway by observation of the interaction of the Xenopus homolog XTcf-3 to β-catenin. β-catenin was identified as a putative binding partner of human Tcf-1 by yeast two-hybrid (Molenaar et al., 1996). They confirmed a physical interaction between *in vitro* translated XTcf-3 and β-catenin by gel retardation assay. XTcf-3 bound an oligonucleotide probe containing the Tcf/Lef consensus site, and addition of *in vitro* translated β-catenin resulted in a supershift. This supershift was not observed using an XTcf-3 N-terminal deletion mutant missing the first 31 amino acids (ΔN). Separate microinjection of mRNAs encoding epitope-tagged XTcf-3 and β-catenin into Xenopus embryos showed nuclear accumulation of XTcf-3 and largely cytoplasmic accumulation of β-catenin by immunohistochemistry. Coinjection of XTcf-3 and β-catenin showed translocation of β-catenin with XTcf-3 to the nucleus, while co-injection of ΔNXTcf-3 and β-catenin showed separation in nuclear and cytoplasmic compartments, respectively. Chloramphenicol acetyl transferase (CAT) reporter assays showed transcriptional activation in the IIAI.6 B cell line upon cotransfection of XTcf-3 and

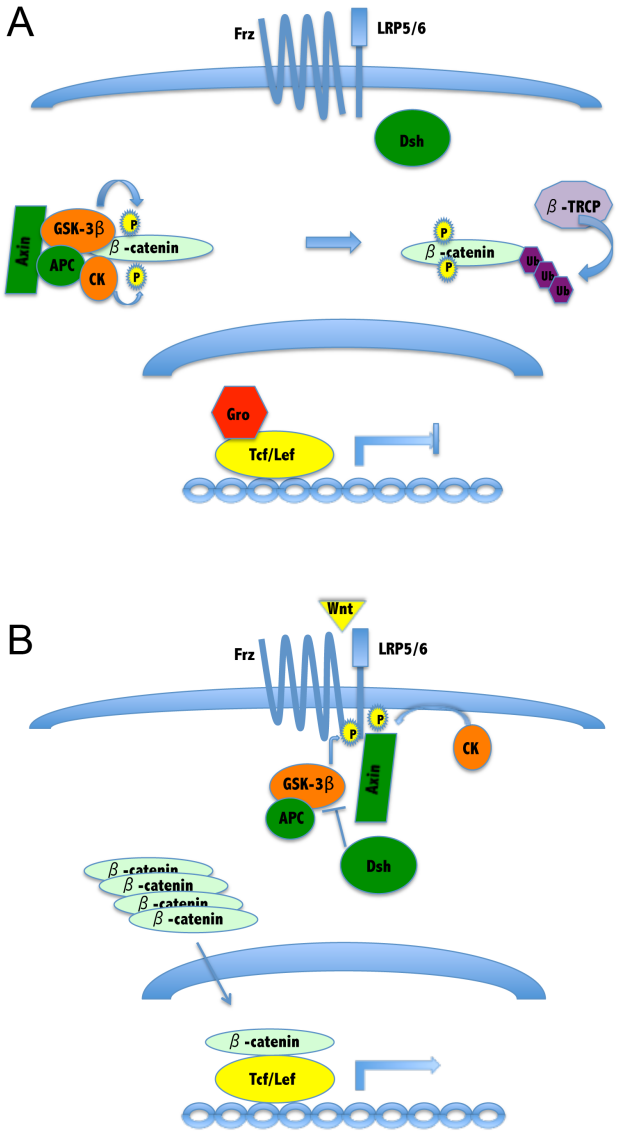

Figure 1.5: **A schematic of Wnt signaling.** A) In the absence of Wnt signaling, cytoplasmic β-catenin is bound by the "destruction complex," which targets β-catenin for ubiquitination and subsequent degradation. Tcf/Lef factors are DNA-binding proteins that are able to repress genes through their interction with Groucho/TLE. B) Binding of Wnt to its receptor triggers the disassembly of the destruction complex, allowing β-catenin to accumulate and translocate to the nucleus to activate transcription of Wnt-responsive genes.

β-catenin, while transfection of XTcf-3 alone failed to activate transcription. Interestingly, ΔNXTcf-3 was shown to inhibit β-catenin induced axis duplication in Xenopus embryos (Molenaar et al., 1996). Importantly, these findings led to the notion that shorter isoforms of Tcf containing a similar deletion in the N-terminal β-catenin binding domain would act as negative regulators or suppressors of transcription (see *Discussion*).

Various gain- and loss-of function models indicate an essential role for Wnt in lymphoid development. Wnt signaling has been demonstrated to be necessary for HSC renewal (Fleming et al., 2008; Reya et al., 2003), and constitutive activation of the key effector β-catenin has been shown to result in a multilineage differentiation block (Kirstetter et al., 2006; Scheller et al., 2006). Conditional deletion of β-catenin in developing thymocytes has been reported to inhibit the DN to DP thymocyte transition (Xu et al., 2003). However, others have reported that reconstitution of congenic mice with BM from donors carrying a deletion of β-catenin did not affect HSC renewal and differentiation to all other hematopoietic lineages (Cobas et al., 2004). Further studies using two independently generated mouse models demonstrated that the concomitant ablation of β-catenin and its potentially redundant family member γ-catenin also had no effect on hematopoiesis, arguing against a role for Wnt signaling in lymphoid development (Jeannet et al., 2008; Koch et al., 2008). However, it should be noted that in one of the doubly deficient models, a shorter (40-50 kD) β-catenin protein product was observed, although this product was incapable of

binding Tcf-1. Interestingly, analysis of a Wnt reporter showed Tcf/Lef-dependent activity in the absence of β- and γ-catenin (Jeannet et al., 2008).

Induction of β-catenin may occur independently of the canonical Wnt signaling pathway. Several studies report the induction of β-catenin downstream of somatically rearranged lymphocyte receptors (Christian et al., 2002; Kovalovsky et al., 2009; Lovatt and Bijlmakers, 2010). In T cells, the activation of β-catenin downstream of the TCR triggers signaling associated with negative selection (Kovalovsky et al., 2009).

1.4 T-cell factor-1 (Tcf-1)

1.4.1 Identification and cloning

Tcf-1, encoded by the *Tcf7* gene, was first named and identified as a T-cell-specific transcription factor binding to a *CD3e* enhancer element. Using a short oligonucleotide probe containing the *CD3e* recognition motif, Tcf-1 was originally cloned from the human T-cell line Jurkat, and three alternative splice variants (i.e. Tcf-1A, Tcf-1B, and Tcf-1C), all about 268-269 amino acids in length and varying only in the C-terminus, were observed. Sequence analysis revealed Tcf-1 to be a member of the then recently described DNA-binding high mobility group (HMG)-box family of proteins HMG-box proteins bind to a consensus site first identified for the sex-determining region (SRY) (van de Wetering et al., 1991).

Murine Tcf-1 was later cloned from the mouse thymic lymphoma cell line EL4 using an oligonucleotide probe containing the cognate DNA binding motif of

human Tcf-1. The isolated clone most closely resembled human Tcf-1B, which was then thought to be the full length Tcf-1. Tcf-1 was indeed found to bind the HMG-box consensus heptamer motif (A/T A/T C A A A G) but was unable to activate transcription by itself (Oosterwegel et al., 1991a; Oosterwegel et al., 1991b; van de Wetering et al., 1991). Unlike Sry-type HMG-box (Sox) protein Sox-4, which was also found expressed in lymphoid tissues, Tcf-1 and Lef-1 are "non-classical" transcription factors in that they can bind DNA but do not have an encoded transactivation domain. Along with Tcf-1 and Tcf-1α (later termed Lef-1), two other HMG-binding homologues were identified in human (HeLa) cells. These homologues were cloned in a PCR-based approach using degenerate primers to amplify genes with sequence similarity to Tcf-1/Lef-1. They were designated Tcf-3 and Tcf-4 and were expressed from the *Tcf7l1* and *Tcf7l2* genes, respectively. These four members together comprise what we now refer to as the Tcf/Lef family (Castrop et al., 1992) (**Figure 1.6**).

Initial studies in both humans and mice indicated the presence of Tcf-1 species of varying lengths, suggestive of alternative splicing. Further studies in humans to detail the Tcf-1 variants confirmed the extensive alternative splicing of *Tcf7*, yielding at least three additional isoforms with high variability in their C-terminal tails. Identification of a novel splice acceptor site internal to exon X revealed the presence of alternate reading frames in this region and alternate usage of the last exon (Castrop et al., 1995a; Mayer et al., 1995; Van de Wetering et al., 1996). Products with significantly different C-terminal peptide

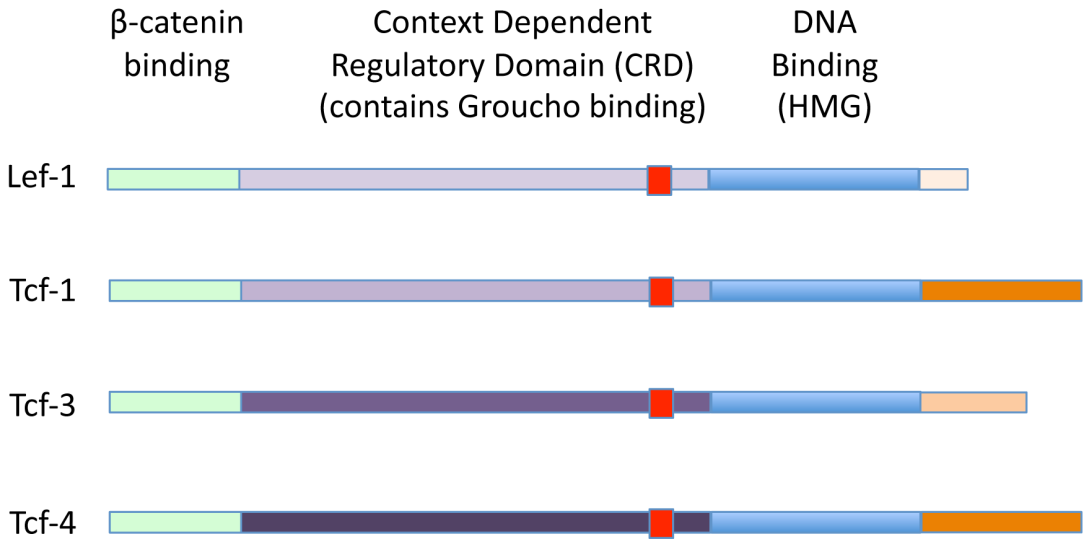

Figure 1.6: **The Tcf/Lef family of transcription factors.** Tcf/Lef family members are identical in their DNA binding domains, which have ability to bend DNA. They share high homology throughout the protein, and can interact with β-catenin and Groucho.

sequences, could have important consequences for Tcf-1 protein interactions and biological function, but the physiological predominance and contributions of the alternate splice forms have not been investigated further. Subsequent cDNA cloning, PCR amplification, and analysis by rapid amplification of 5' cDNA ends revealed the presence of an alternative upstream promoter, which extends the N terminus by 116 amino acids. It is of important note that the original Tcf-1 clones identified in murine thymic lymphoma cell lines were products of the downstream promoter and therefore missing the first 116 amino acids, which contain the β-catenin binding domain (See *Discussion*).

1.4.2 Expression and function in the thymus

In addition to its expected interaction with the murine *CD3e* enhancer, sequence-specific binding was also observed within the T-cell receptor TCRα, β, and δ enhancers, further implicating Tcf-1 in T-cell development (Oosterwegel et al., 1991a; Oosterwegel et al., 1991b). High-stringency Northern blotting of mRNA prepared from various tissues determined the expression of murine Tcf-1 to be confined to the thymus and spleen. *In situ* hybridization showed Tcf-1 expression in the spleen to be restricted to the periarteriolar lymphocyte sheaths (PALS), with no Tcf-1 expression in red pulp, marginal zone, or B cell follicles, suggesting Tcf-1 to be specific for T cells. Subsequent Northern blotting of various lymphoid cell lines showed the greatest Tcf-1 expression in mature T-cell lines, although Tcf-1 was also present in prothymocytes and remained absent in pro/pre-B-cell, B-cell, and fibroblast lines (Oosterwegel et al., 1993). Generation of an anti-Tcf-1 antibody in Tcf-1-deficient mice immunized with a protein fusion

of the C-terminal half of human Tcf-1A and mannose binding protein (MBP), allowed for the detection of Tcf-1 protein. These studies demonstrated the presence of Tcf-1 protein only in cell lines of T-cell origin and the localization of Tcf-1 to the nucleus (Castrop et al., 1995b).

To investigate the role of Tcf-1 in T-cell development further, two strains of mutant mice were generated carrying different germline mutations in Tcf-1. The first of these mutants, $Tcf^{\Delta V}$, contains a short deletion in what was previously termed exon V, which is a conserved region in *Drosophila*. The second mutant, $Tcf^{\Delta VII}$, contains a deletion in what was previously termed exon VII (exon 9 in Ensembl 201), which encodes the DNA-binding HMG-box. Heterozygous mice carrying only one mutant allele had the wildtype phenotype. Mice homozygous for the deletion in exon V showed a 2- to 5-fold reduction in thymocyte numbers, while mice homozygous for the deletion in exon VII showed a dramatic 10-fold reduction in thymic cellularity. Although the data were not shown, Northern blot analysis of mRNA obtained from these two mouse models indicated the former to express a *Tcf-1* mRNA with an in-frame deletion of exon V whereas the latter mouse contains no *Tcf-1* transcript. The $Tcf^{\Delta VII}$ mice will henceforth be referred to as $Tcf\text{-}1^{-/-}$ mice. In addition to hypocellularity, the thymi of these mice were observed to have an increased percentage in ISP while maintaining a normal percentage of DN cells and a decreased percentage of DP cells. While cell numbers were also decreased in secondary lymphoid organs (i.e. spleen and lymph node), cells appeared functionally normal insofar as they maintained responsiveness to alloantigen and concanavalin A (conA). B cell and $NK1.1^{+}$ cell

numbers were reported to be unaffected. The authors concluded that these mice had a partial developmental block at the ISP stage (Verbeek et al., 1995).

In the periphery, intestinal γδ-T cells were found to be reduced in *Tcf-1*$^{-/-}$ mice as compared to wildtype, but this reduction of γδ-T cells was specific for the intestine since thymic and splenic γδ-T cell populations appeared to be unaffected. NK1.1^{+} T cells in the liver were also found to be reduced in *Tcf-1*$^{-/-}$ mice with CD4^{+} and DN NK1.1^{+} TCRβ^{+} cells almost absent, while CD8^{+} NK1.1^{+} TCRβ^{+} cell ratio was increased. This phenomenon was also observed in the thymi of *Tcf-1*$^{-/-}$ mice (Ohteki et al., 1996).

As with *Gata3*, analysis of *Tcf7* expression in FT suggested that Tcf-1 was likely involved in the earliest stages of T-cell development in the thymus. Generation of polyclonal anti-Tcf-1 antibodies from rabbits immunized with polypeptides of the murine Tcf-1B clone allowed immunocytochemical staining, which showed that while day 12 FT was only about 30% positive for Tcf-1, day 16 FT was almost 100% positive, and expression kinetics of Tcf-1 preceded that of CD3 (Hattori et al., 1996).

Clevers and colleagues later reported that *Tcf-1*$^{-/-}$ mice have a prominent DN1 population in contrast to an almost complete absence of DN2 cells. While the frequency of ISP was higher than in control, they were greatly reduced in Tcf-1-deficient mice, but by 6 months of age, ISP cells in *Tcf-1*$^{-/-}$ mice were completely absent, suggesting an age-dependent role for Tcf-1. Analysis of the DNA content of DN progenitors suggested that the reduction of thymic cellularity in *Tcf-1*$^{-/-}$ mice was due to the absence of cycling cells (Goux et al., 2005;

Schilham et al., 1998). Furthermore, while *Tcf-1*$^{-/-}$ DN3 and DN4 cells were able to proliferate normally as assessed by BrdU incorporation, AnnexinV staining indicated that they were more apoptotic (Goux et al., 2005). Th cell function in peripheral T cells was found to be normal as determined by quantification of IgG in response to Semliki Forest virus (SFV) infection. Moreover, these cells displayed a more activated "phenotype," as described by being CD44^{+}CD62L^{lo} (Schilham et al., 1998).

Transgenic mice were generated to express Tcf-1 with or without the β-catenin binding domain (p45 or p33, respectively) and were crossed to the *Tcf-1*$^{-/-}$ mice to observe whether expression of the transgene could rescue Tcf-1 deficiency. The p45 (containing the β-catenin binding domain) transgenic was able to partially restore thymic cellularity, the DP phenotype, and survival, whereas p33 could not. Co-expression of both p45 and p33 restored the mutant phenotype further (Ioannidis et al., 2001).

In opposition to their role as transcriptional activators, Tcf/Lef proteins have also been shown to be transcriptional repressors. The same yeast two-hybrid (from which the data was not shown) that revealed an interaction between Tcf and β-catenin also indicated a putative interaction between Tcf and Groucho (Grg; also referred to as transducin-like enhacer of split, TLE), which had been known to repress transcription by recruitment of histone deacytelase 1 (HDAC1) (Brantjes et al., 2001; Cavallo et al., 1998; Roose et al., 1998). While much of the work on Tcf-1 has focused on its activating Wnt/β-catenin-dependent function, the finding that Tcf-1 could interact with other transcriptional regulators and

chromatin modifiers highlighted β-catenin-independent activity that remains poorly understood and has lacked further functional and physiological exploration. While the β-catenin-independent functions had already been suggested by the identification of abundantly expressed, naturally occurring isoforms lacking the β-catenin-binding domain, these studies indicated an important repressive role for Tcf-1 that had been ignored (see *Discussion*).

1.4.3 HMG box proteins and potential redundancy of Tcf-1 and Lef-1 factors

Tcf-1 and Lef-1 have been speculated to have redundant functions as they have identical DNA binding domains and are highly homologous throughout the protein. Indeed fetal cells from the combined *Lef-1*$^{-/-}$*Tcf-1*$^{\Delta V/\Delta V}$ have an exacerbated phenotype in giving rise to T-cells when compared to cells from the hypomorphic *Tcf-1*$^{\Delta V/\Delta V}$ alone. It should be noted that the defect exhibited by the more widely used *Tcf-1*$^{\Delta VII/\Delta VII}$ is comparable to that observed in the double mutant (Held et al., 2003).

Additionally, other HMG-box factors such as Sox4, Sox13, and Tox might also be able to recognize similar sites. The Sox and Tox factors are “classical” transcription factors in that they contain their own transactivation domains. While Tcf-1 is expressed in αβ T cells along with fellow HMG proteins Lef-1 and Sox4, Sox13 is uniquely expressed in γδ T cells (Melichar et al., 2007).

1.4.4 Tcf-1 expression and function in peripheral T cells

Tcf-1 may have a role in Th differentiation. For example, one study reported the identification of a Tcf-1 consensus-binding site upstream of the

Gata3-1b promoter. Additionally, *Tcf-1*$^{-/-}$ mice show impaired IL-4 but enhanced IFN-γ production (Yu et al., 2009). In contrast, another study reported the preferential downregulation of the Tcf-1 short-isoforms in naïve CD4^{+} T cells cultured in the presence of the Th2-polarizing cytokine IL-4 in a STAT6-dependent pathway (Maier et al., 2011).

In addition to a role in Th2 cells, Tcf-1 has been implicated in Th17 development and maintenance. *Tcf7* expression is induced *in vitro* in Th17 polarized cells, and expression of *Tcf7* remains high in these cells *ex vivo* when recovered from adoptive transfer (Muranski et al., 2011). In contrast, others report a role for Tcf-1 in direct repression of the IL-17 gene. When compared to control, Tcf-1 deficient mice show enhanced Th17 differentiation when cultured in Th17-polarizing conditions, and *Tcf-1*$^{-/-}$ mice have increased disease severity when induced with experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis known to be dependent on Th17 cells. Tcf-1 can bind the IL-17 gene directly, and hyperacetylation and trimethylation, which are marks of open, active chromatin, were observed to increase at this regulatory region in *Tcf-1*$^{-/-}$ mice, arguing for a direct role for Tcf-1 in IL-17 repression (Ma et al., 2011; Yu et al., 2011).

Tcf-1 and the Wnt signaling pathway have also been implicated in the formation of memory CD8 T cells. Microarray analysis comparing the signatures of naïve T cells (T_N) with antigen-experienced effector memory (T_{EM}) and central memory (T_{CM}) cells has indicated that Lef-1 and Tcf-1 are downregulated upon antigen encounter but then are upregulated in T_{CM} (Willinger et al., 2006).

Interestingly, short isoforms (lacking the β-catenin binding domain) of Tcf-1 decreased in antigen-experienced CD8 T cells. Further study indicated that inhibition of GSK-3β led to impaired effector CD8 cell differentiation, while promoting the development of so-called stem cell memory cells (T_{SCM}, $CD44^{lo}CD62L^{hi}Sca\text{-}1^{hi}CD122^{hi}Bcl\text{-}2^{hi}$) that exhibited enhanced recall and anti-tumor response (Gattinoni et al., 2009). Tcf-1 deficiency resulted in impaired CD8 central memory development, although CD4 memory seemed unaffected. The requirement for Tcf-1 was β-catenin dependent and was associated with *Eomes* expression (Jeannet et al., 2010; Zhou et al., 2010).

These studies provided evidence for a role for Tcf-1 in mature effector cells. The common requirements of CD8 memory cells and NK cells for *Eomes* and signaling through IL-15, and the *Eomes*-dependent defect observed in the CD8 memory compartment of *Tcf-1*$^{-/-}$ mice encouraged further speculation about the role of Tcf-1 in early NK development.

1.4.5 Tcf-1 expression and function in NK cells

Tcf-1 expression has been detected in both human and murine NK cells (Held et al., 1999; Toor et al., 2001). While initial reports of the *Tcf-1*$^{-/-}$ phenotype indicated no apparent defect in hepatic NK cells, further study indicated deficiency of BM NK cells, and clonal expression of Ly49A was significantly reduced in the NK repertoire. Interestingly, despite multiple reports suggesting the lack of an obvious phenotype in *Tcf-1*$^{+/-}$ mice, the effect on Ly49A acquisition by Tcf-1 was reported to be dosage-dependent, with heterozygous mice

exhibiting intermediate expression of Ly49A. Two Tcf-1 consensus binding sites were identified within 100 base pairs of the transcriptional start site (Held et al., 1999). Interestingly, one site was found to be activating while the other was repressive. Tcf-1 additionally regulated other Ly49 receptors, presumably through trans-acting elements (Ioannidis et al., 2003; Kunz and Held, 2001).

The defect in Ly49A acquisition provided a clue to a role for Tcf-1 in developing BM NK cells as Ly49A expression is acquired at the iNK stage. Since little is known as to the transcriptional control of the developing NK cell, these early findings needed to be pursued further.

1.4.6 Tcf-1 expression in disease

While Tcf-1 expression is widespread in developing embryos, Tcf-1 was initially reported as being expressed only in cells of the T lineage in the adult. However, there is increasing evidence for the expression of Tcf-1 in other cell types, especially of its aberrant expression and regulation during disease. Many members of the canonical Wnt signaling pathway have been shown to be upregulated in many types of cancer (Nusse, 2005), and Tcf-1 is no exception. Ectopic overexpression of *Tcf7* mRNA was found in colorectal and mammary tumors, however basal transcription of *Tcf7*, a target of β-catenin/Tcf-4 signaling, was detected even in normal colon cells (Mayer et al., 1997; Roose et al., 1999). While Tcf-1 activity was reportedly upregulated in epithelial tumor cells as measured by CAT reporter assays, the short (β-catenin-independent) isoform was the predominant splice variant detected (Mayer et al., 1997; Roose et al., 1999). *Tcf-1*$^{-/-}$ mice develop colon and mammary tumors suggesting a role for

Tcf-1 as a tumor suppressor (Roose et al., 1999). The dominant negative isoform of Tcf-1 was found equally distributed between nuclear and cytoplasmic compartments in normal colon cells, but Tcf-1 protein was predominantly cytoplasmic in colon cancer cells. Additionally, short Tcf-1 isoform expression decreased while long (activating) isoform expression increased (Najdi et al., 2009).

In human disease, two SNPs within the *Tcf7* locus were identified with association to type I (juvenile onset, autoimmune) diabetes. *Tcf7* was shown to be expressed in the pancreas, although the authors did not make any distinction as to which cell type(s) expressed it. *Tcf7* expression was shown to decrease upon treatment with insulin in rats (Columbus et al., 2010).

The identification of tissues in non-lymphoid organs in which *Tcf7* is expressed, and the correlation of *Tcf7* expression with disease highlight the importance of studying the function and regulation of the evolutionarily conserved Tcf-1 protein. While the role of Tcf/Lef proteins as mediators of stem-cell renewal downstream of the Wnt-signaling pathway have provocative implications for cancer, the differential expression of Tcf-1 short and long isoforms found in normal and diseased colon epithelia indicate an important biological role for β-catenin-independent or repressive functions of Tcf-1 that should be explored.

While much study been done to characterize the phenotype of Tcf-1 deficient mice, its functions in lymphoid development remain poorly understood. While specific defects had been noted at the ISP and DN2 stages, more recent

information resolving the heterogeneity of early thymic subsets and the identification of hematopoietic progenitor intermediates between the HSC and the TSP demanded an updated analysis of the Tcf-1 defect. Additionally, while many factors involved in the instruction of the lymphoid fate have been described, the placement of Tcf-1 within this regulatory network had not been established. Finally, further analysis is necessary to understand the molecular role of Tcf-1 and potential Tcf-1 responsive effectors in early precursors. These together would aid in improved understanding of normal lymphoid development and may provide insights to the role of Tcf-1 in disease.

CHAPTER II:

MATERIALS AND METHODS

2.1 Animals

All mice were kept in the animal facilities of the University of Chicago according to protocol no. 71880 approved by the Institutional Animal Care and Use Committee. *Tcf-1*$^{-/-}$ mice on the C57BL/6 (B6) background were obtained from Dr. Hans Clevers and Dr. Frank Staal (Verbeek et al., 1995). We have previously reported the generation of the pre-TCR-alpha-HuCD25 reporter mice (Gounari et al., 2002). The generation of *Notch1*$^{fl/fl}$, *Notch2*$^{fl/fl}$ and MxCre mice were described in (de Boer et al., 2003; McCright et al., 2006; Radtke et al., 1999).

2.2 Monoclonal antibodies and flow cytometry

Multicolor-FACS stainings were performed for analysis and cell sorting of primary thymocytes on LSRII, FACSCanto or FACSAria instruments (BD Biosciences). Antibodies were from BD Biosciences or eBioscience: CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (6D5), CD25 (PC61.5), CD44 (1M7), B220 (RA3-6B2), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), (53-2.1), CD122 (5H4), c-kit (2B8), Gr1 (RB6-8C5), Sca-1 (D7), TCRb (H57-597), TCRgd (eBioGL3), NK1.1 (PK136), DX5 (DX5), Ter119 (Ter119). Biotinylated antibodies were detected with streptavidin–PE-Cy5.5 or eFluor780. Annexin V-PE labeling kit (BD Biosciences) was used according to the manufacturer's instructions. Data were analyzed in FlowJo software (Tree Star).

2.3 Bone marrow chimeras

Lethally irradiated (950 rad; Gammacell 40) CD45.1^{+} C57BL/6 mice (host) were

injected retro-orbitally with a 1:1 mixture of FACS-sorted host and *Tcf-1*$^{-/-}$ donor lineage (B220, CD3, CD8, CD4, CD11b, CD11c, CD19, NK1.1, Ter119) negative bone marrow (1×10^6 cells per mouse). Bactrim was added to the drinking water for the time of observation (4-10 weeks).

2.4 OP9-DL1 cell culture conditions

A 1:1 mixture of 10^4 FACS-sorted CD45.1$^+$ WT and CD45.2$^+$ *Tcf-1*$^{-/-}$ cells from corresponding progenitor subsets were transferred to a 24-well plate seeded with OP9-DL1 24 hours prior to co-culture to be ~60% confluent. Progenitors were co-cultured as described previously (Garbe et al., 2006). Cultures were carried out for 10 days before analysis.

2.5 In vivo BrdU-incorporation assay

Mice were injected retro-orbitally with 0.5 mg BrdU (Sigma) per 5 g of body weight 2 h prior to analysis of BM and thymic subpopulations by flow cytometry. BrdU staining was performed with the FITC-BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions.

2.6 RNA extraction and quantitative real-time RT-PCR

Cells were lysed and RNA was extracted with the RNeasy Micro kit (Qiagen). cDNA was prepared with the SuperScript-III RT kit (Invitrogen). Quantitative PCR was performed on an ABI7300 machine (Life Technologies) relative to *Gapdh* expression using TaqMan Gene Expression Assays from Applied Biosystems

(Life Technologies). Data were analyzed according to the relative $\Delta\Delta C_T$ method. Experiments were done in triplicate.

2.7 ChIPseq analyses

ChIP-Seq for Notch1 and CSL was performed as described (Wang et al., 2011) using sheared chromatin from T6E cells. Data are available through the Gene Expression Omnibus (accession# GSE29600). For Tcf-1 ChIP-seq, 10^8 WT thymocytes were formaldehyde-fixed and sonicated to an average size of 300 bp. Tcf-1 antibodies (a kind gift of Hiroshi Kawamoto) coupled to Protein G Dynabeads (Life Technologies) were incubated o/n with sheared chromatin. IP and input samples were de-crosslinked o/n at 65°C in elution buffer (50 mM Tris HCl (pH8)/ 10 mM EDTA/ 1% SDS/ 0.3M NaCl). DNA was extracted with Phenol/Chloroform, quantified and quality-checked for enrichment of known target regions of Tcf-1 by realtime PCR. ChIP-seq libraries were prepared from 10 ng of IP'ed material according to Illumina ChIP DNA library preparation kit. After deep sequencing on Illumina Genome Analyzer II, 32bp reads were mapped to mouse genome version mm9 using the ELAND alignment tool in GApipeline accepting no more than two mismatches. Uniquely aligned tags were selected, converted to bed files and after read normalization were analyzed by the peak calling software MACS (Zhang et al., 2008). The algorithm was applied using a 2d sliding window across the genome to find candidate peaks with significant tag enrichment according to Poisson distribution at a default P-value of 10^{-5} with input control data.

2.8 Gene expression microarrays

Approximately 1000 ETPs were sorted directly into 100ml lysis buffer and RNA was extracted as recommended (Arcturus PicoPure RNA Isolation kit, Life Technologies, Foster City, CA). RNA quality and concentration were estimated using the Bioanalyzer Pico Chip and RNA 6000 Pico Assay reagents (Agilent, Santa Clara, CA). Average yield from 1000 cells was 3 ng of total RNA. All material was amplified using Ovation Pico WTA System (NuGen, San Carlos, CA). Labeling, fragmentation and hybridization to Mouse Genome 430 2.0 Arrays were done according to the manufacturer's instructions (Affymetrix Inc., Santa Clara, CA). Comparative analysis of gene expression profiles from WT and mutant progenitors was performed by the Bioconductor limma package (Smyth, 2004). GO analysis of target genes was conducted using the Functional Annotation Tool of the DAVID software.

2.9 EMSA

Nuclear extracts were prepared according to standard protocols. In brief, cell homogenates were centrifuged and resuspended in a hypotonic buffer containing 0.4% (vol/vol) NP-40 followed by a buffer with a higher salt concentration before the supernatant (nuclear proteins) was collected. Before the addition of biotin-labeled DNA probe, 5 mg nuclear extract or 0.5 mg purified CSL protein was incubated for 20 min on ice in 20 ml reaction buffer containing 1x binding buffer, 1 mg double-stranded poly(dI:dC), 2.5% (vol/vol) glycerol, 0.05% (vol/vol) NP-40

and 1 mg BSA. Samples were incubated for 20 min at room temperature with the following biotinylated probes (20 fmol/each):

WT 5'–CCGAGACGTAGTATTCCCACCACACGCCACCTTC–3',

Mutant 5'-CCGAGACGTAGTATTAAAACCACACGCCACCTTC–3'.

For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides was added. Protein-DNA complexes were separated by electrophoresis through 6% non-denaturing TBE gels (Life Technologies) and were visualized with a LightShift Chemiluminescent EMSA kit (Pierce).

2.10 Plasmids and molecular techniques

Tcf-1 was cloned from cDNA of wt mouse thymocytes using the following primers, engineered to contain a 5' BglII site and 3' EcoRi site:

Tcf7_201_wtF, TAGCAGATCTATGCCGCAGCTGGACTCGGG

Tcf7_201_R, CGATGAATTCCTAGAGCACTGTCATCGGAAGGAAC

Likewise, Tcf-1 carrying a deletion in the β-catenin binding domain (aa1-69) was cloned with the following primer and the same reverse primer listed above:

Tcf7_201_deltacatF,TAGCAGATCTATGTACAAAGAGACTGTCTACTCTGCCTT

Tcf-1 WT and Tcf-1ΔβCBD were cloned into the multiple cloning site of MigR1 adapted from (Pear et al., 1998)

The TOP-eGFP and FOP-eGFP reporters were a kind gift from I. Weissman.

2.11 Retroviral transduction of BM progenitors

Lentiviral particles were packaged by contransfection of VSVG CMV into 293T

cells using the FuGene (Roche) reagent according to manufacturer protocol. Retroviral particles were packaged with the Platinum-E (Plat-E) packaging cell line using FuGene according to manufacturer protocol.

For transduction, non–tissue culture plates (Corning) were coated overnight at 4 °C with RetroNectin (25 mg/ml, Takara), then were blocked for 30 min with 2% (wt/vol) BSA in PBS and finally incubated for 4h at 37 °C with retroviral supernatant. After coating, viral supernatant was removed and fetal Lin^- c-kit^+CD27^+ FL or BM LSKs were added immediately in OP9 coculture medium as described below, with 5 ng/ml each of Flt3L, IL-7 and SCF. Cells were then cultured on the virus-coated plates for 48 hours for transduction.

Progenitors were first cultured on tissue culture treated plates (Corning) for 24 hours in OP9 coculture media with 20 ng/ml of IL-3, 50 ng/ml of IL-6, 50 ng/ml of SCF, 5 ng/ml of Flt3Land 5 ng/ml of IL7. Then, cells were transferred onto virus-coated plates and cultured for transduction for 48. hours with the same cytokines and concentrations.

CHAPTER III:

RESULTS I: TCF-1 IN THE BONE MARROW

While HSCs constitute the most studied adult stem cell system, the precise mechanisms underlying HSC proliferation, survival, and differentiation remain elusive. Wnt signaling has been demonstrated to be necessary for HSC self-renewal (Nemeth and Bodine, 2007; Reya et al., 2003), and constitutive activation of the key effector and transcriptional activator β-catenin in HSCs has been shown to result in a multilineage block of hematopoietic development (Kirstetter et al., 2006; Scheller et al., 2006). Paradoxically, conditional deletion of β-catenin alone or β- and γ-catenin combined indicated no effect on HSC renewal and hematopoiesis (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). However, transient reporter assay analysis indicated Tcf/Lef-dependent activity even upon ablation of β- and γ-catenin (Jeannet et al., 2008). Despite the inconclusive data on Wnt signaling in hematopoietic development, as reviewed in the *Introduction*, the downstream factors Tcf-1 and Lef-1, which, together with β-catenin, promote expression of Wnt-responsive genes, have long been established to play critical roles in the development of lymphocytes (reviewed in *Introduction*). The complexity of Wnt signaling regulation suggested that a simpler approach to study the involvement of Wnt in lymphoid development might be to revisit the role of Tcf-1 in early lymphoid development.

Advances in our current understanding of hematopoiesis and the characterization of novel progenitor subsets prompted our interest to review the role of Tcf-1 (product of the *Tcf7* gene) in early hematopoietic development in mice. We first sought to carefully map the Tcf-1 expression pattern in precursor populations from the bone marrow. To this end, we performed quantitative PCR

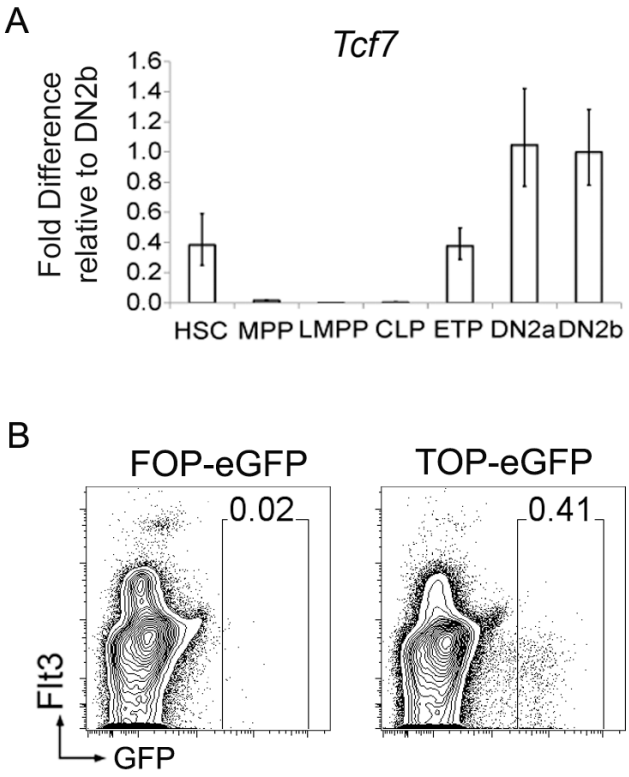

Figure 3.1: **Tcf-1 expression and activity in the BM and thymus. A.** HSC, MPP, LMPP, and CLP as defined in Figure 1.1 were sorted from the BM of WT mice. Similarly ETP, DN2a and DN2b cells were sorted from the thymus of WT mice. RNA was extracted and analyzed by q-PCR. *Tcf7* expression was normalized to *Gapdh*. Histograms show relative expression levels in the various subsets normalized to the levels of DN2b. Error bars representative standard deviation from technical triplicates. Data are representative of two experiments. (Germar et al., 2011) **B.** LSK cells infected with either reporter (TOP-eGFP) or control (FOP-eGFP) virus were used to reconstitute lethally-irradiated, congenic hosts. Recipient BM was assessed for GFP activity 10 weeks after transfer. Dot plots shown were gated on Lin^- cells. Data are representative of two experiments.

(qPCR) to measure *Tcf7* levels in the HSC ($Lin^-Scal^{hi}c\text{-}kit^{hi}Flt3^-$), MPP ($Lin^-Sca1^{hi}c\text{-}kit^{hi}Flt3^{lo}$), LMPP ($Lin^-Sca1^{hi}c\text{-}kit^{hi}Flt3^{hi}$) and CLP ($Lin^-Sca1^{lo}c\text{-}kit^{lo}Flt3^+IL\text{-}7Ra^+$). We detected *Tcf7* expression in the HSC (**Figure 3.1A**), consistent with reports suggesting that Wnt is important for self-renewal in HSCs (Fleming et al., 2008; Reya et al., 2003), but *Tcf7* expression level declined in subsequent BM progenitors and was largely undetectable in MPP, LMPP, and CLP cells (**Figure 3.1A**).

Tcf7 expression levels may not reflect transcriptional activity. To assess whether signaling via Tcf/Lef occured in early hematopoietic development *in vivo*, we employed the TOP-eGFP lentiviral reporter system, in which enhanced green fluorescent protein is expressed under the control of a promoter containing a multimerized Tcf/Lef binding motif (Reya et al., 2003). The system is complemented by a mutant construct (FOP-eGFP) that contains mutated Tcf/Lef binding sites rendering it insensitive to Tcf/Lef activation. Lin^- BM cells were transduced with the TOP-eGFP or control FOP-eGFP reporter constructs, and transduced cells were used to reconstitute lethally-irradiated, congenic ($CD45.1^+$) hosts. Recipient tissues were examined for reporter activity 6 weeks after transfer. While eGFP expression was not detected in donor cells ($CD45.2^+$) transfected with FOP-eGFP or in host cells, some Tcf/Lef activity was detected in Lin^- cells, although individual progenitor populations were not assessed (**Figure 3.1B)**.

We reasoned that if the relatively low levels of Tcf-1 expression and activity observed in the HSC were to reflect a functional requirement for the

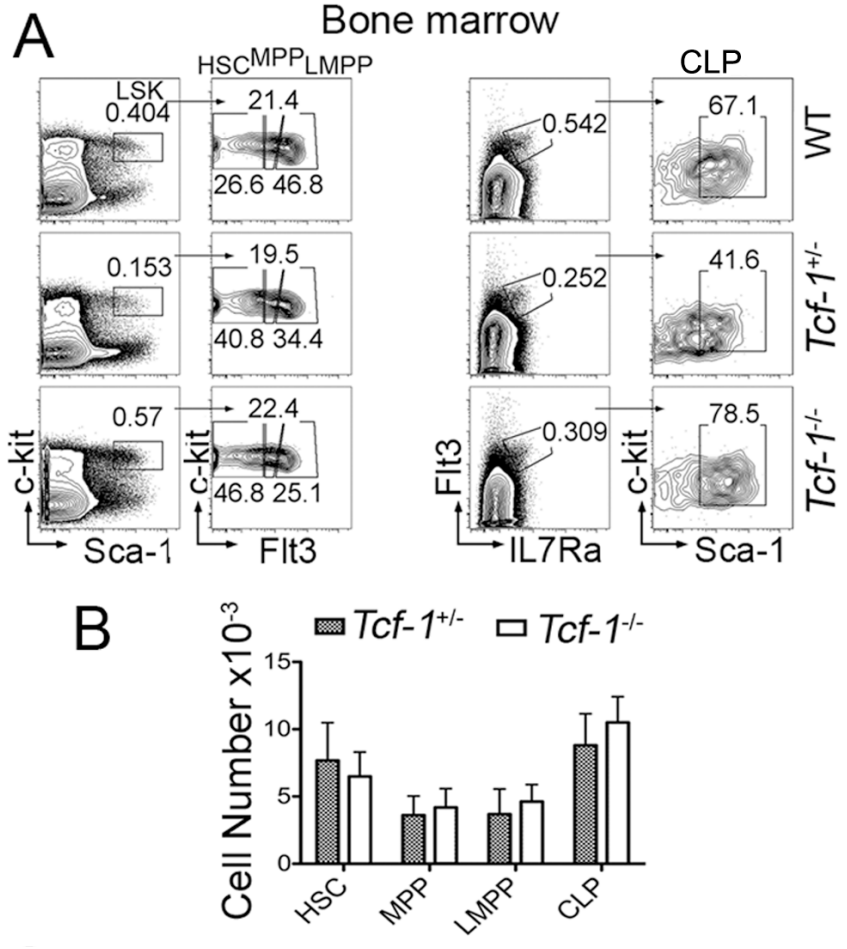

Figure 3.2: BM progenitors in *Tcf-1*$^{-/-}$ mice are normal in number. A. Analysis of gated Lin$^-$ BM cells is shown. c-kit versus Sca-1 profiles show the gating for LSK progenitors. c-kit versus Flt3 profiles show the HSC, MPP and LMPP subsets as indicated. Flt3 versus IL7Rα profiles show the gating for Lin$^-$ Flt3$^+$IL7Rα^+ cells and c-kit versus Sca-1 plots depict the CLP. Plots are representative of 3 experiments. **B**. Bar histograms show absolute numbers of HSCs, MPPs, LMPPs, and CLPs in the indicated mice. Error-bars show standard deviation where N=3-6 for each group. Heterozygous mice were used as controls as they were phenotypically indistinguishable from wildtype.

protein, then ablation of Tcf-1 would affect all subsequent developmental subsets. To establish whether Tcf-1 ablation negatively impacted bone marrow progenitors, we next evaluated prethymic hematopoietic development in *Tcf-1*$^{-/-}$ mice. Interestingly, an analysis of subset distribution, cellularity, and proliferation properties of HSC, MPP, LMPP, and CLP showed that these populations were not detectably impacted by Tcf-1 deficiency (**Figures 3.2** and **3.3**). Heterozygous mice have a phenotype indistinguishable from wildtype. Although Tcf/Lef family factors have been suggested to play redundant roles in various developmental systems (Galceran et al., 1999; Held et al., 2003), further analyses showed no evidence of compensatory expression of Lef-1, Tcf-3 or Tcf-4 mRNA in the absence of Tcf-1 as analyzed by qPCR of BM progenitors as compared to control (data not shown).

Taken together, these observations suggest that Tcf-1 is dispensable for the development of bone marrow hematopoietic progenitors, and that this is not a result of compensation by other Tcf/Lef family members. This raises the possibility that the perceived role for Wnt signaling in HSC self-renewal involves non-canonical mediators (transcriptional modulators other than Tcf/Lef family members) in the nucleus. Likewise, it cannot be ruled out that a defect in long-term potential exists but does not alter cellular output of early progenitor populations in the absence of challenging the system. This question could further be explored in future studies by performing serial transplantation experiments to assess the long-term reconstitution ability of *Tcf-1*$^{-/-}$ HSC.

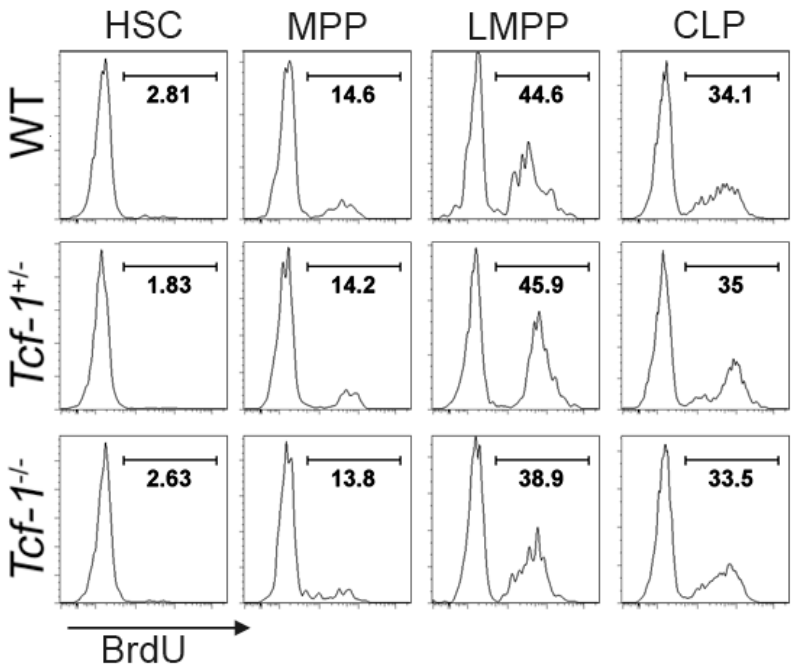

Figure 3.3: BM progenitors in *Tcf-1*$^{-/-}$ mice show no defect in proliferation. A. Cycling of the indicated progenitors was compared in the indicated mouse strains by staining for BrdU. All mice were injected intraperitoneally with BrdU two hours before harvesting the cells for surface markers followed by BrdU staining. Histogram plots show BrdU staining in the indicated gated populations and mouse strains. Numbers in the plots represent the fraction of BrdU^{+} cells in each population. The figure shows cells harvested 2 hours after BrdU injection. This is one representative experiment out of three. The modest decrease shown here for the LMPP was not reproducible. (Germar et al., 2011)

CHAPTER IV:

RESULTS II: TCF-1 IN T-CELL DEVELOPMENT

Earlier reports demonstrated that ablation of Tcf-1 caused an age-dependent degeneration of T-cell development (Schilham et al., 1998). These studies showed that the DN1 population, thought to represent the earliest thymocyte subset at the time, was abundantly present in *Tcf-1*$^{-/-}$ mice, while the subsequent DN2, DN3, double positive (DP), and single positive (SP) populations were dramatically reduced. It is now appreciated that the DN1 subset is a highly heterogeneous population that can be subdivided into at least five subsets (DN1a-e) (Porritt et al., 2004). The c-kit expressing DN1a and DN1b subsets contain the most potent T-cell progenitors and are otherwise known as early thymic progenitors (ETPs) (Allman et al., 2003). However, T-cells can also be derived from the atypical DN1c-e subsets, although inefficiently (Porritt et al., 2004). We next set out to carefully examine the impact of Tcf-1 ablation on early thymic progenitor subsets.

4.1 Tcf-1 is required for T-cell development starting at the ETP stage.

Our expression analysis revealed a marked increase in *Tcf7* transcript upon entry into the thymus. While *Tcf7* expression was low in BM LMPP and CLP stages, ETP (Lin^{-}CD44^{+}c-kit^{hi}CD25^{-}) showed a sharp upregulation of *Tcf7*, which remained high throughout the DN stages (**Figure 3.1**).

An assessment of Tcf/Lef activity using the TOP-eGFP lentiviral reporter system (described in *Chapter III*) confirmed the presence of Tcf/Lef-dependent transactivation in total thymocytes (**Figure 4.1A**). We observed reporter activity

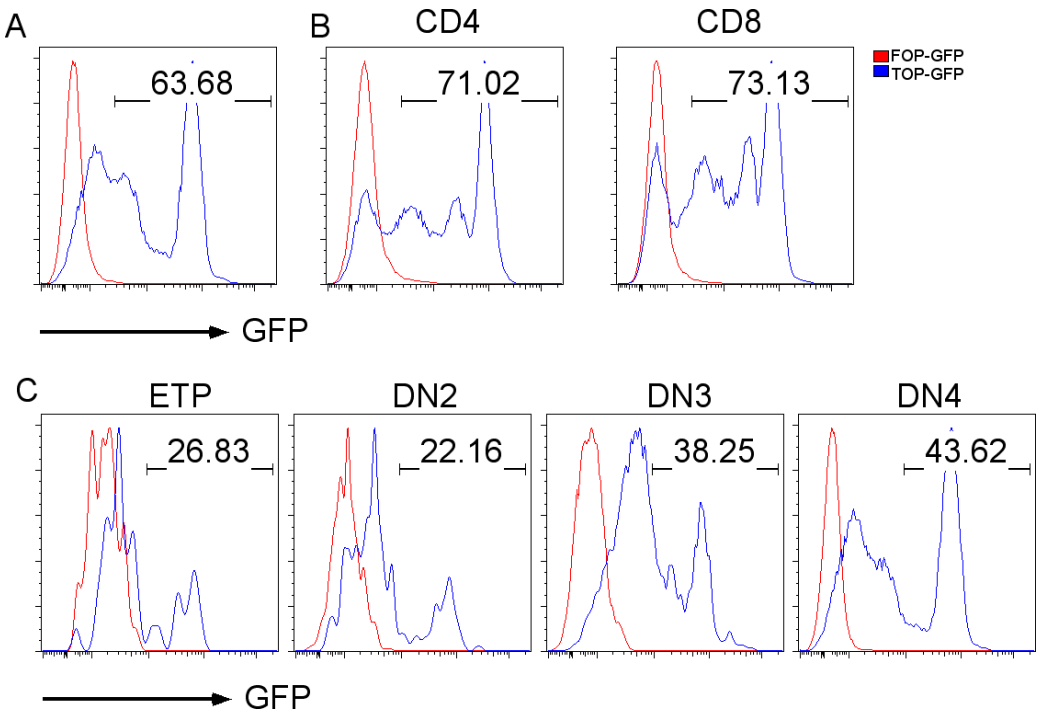

Figure 4.1: Tcf-1 activity in thymus and spleen. LSK cells infected with either reporter (TOP-eGFP) or control (FOP-eGFP) lentivirus were used to reconstitute lethally-irradiated, congenic hosts. Recipient total thymocytes (**A**), $CD4^+$ and $CD8^+$ cells in the spleen (**B**), and thymic progenitors (**C**) were assessed for GFP activity 10 weeks after transfer. Histograms of reporter activity (blue) are shown overlaid against control (red) cells. Data are representative of two experiments.

in the ETP and throughout the DN stages, with a slight increase in the distribution of reporter positive cells leading to the DN4 (**Figure 4.1C)**. The spleen exhibited four levels of Tcf/Lef activity seen in both $CD4^+$ and $CD8^+$ cells (**Figure 4.1B**). This is consistent with reports for peripheral $CD8^+$ T-cells showing the highest level of *Tcf7* expression at the in naïve T cells, downregulation upon antigenic encounter, and then upregulation in memory cells (Gattinoni et al., 2009; Jeannet et al., 2010; Willinger et al., 2006; Zhou et al., 2010); however, since no additional surface markers were included in these analyses, this speculation remains to be verified.

In light of these new findings, we re-analyzed the earliest thymic subpopulations in *Tcf-1*$^{-/-}$ mice, including the classical DN1 ($Lin^-CD25^-CD44^+$) (**Figure 4.2A)**, ETP **(Figure 4.2B)**, and the DN1a-e subsets **(Figure 4.3A)**. Importantly, these analyses showed that *Tcf-1*$^{-/-}$ thymi had a previously unappreciated 100-fold reduction in absolute DN1 numbers and a dramatic 300-fold reduction in ETP compared to *Tcf-1*$^{+/-}$ or WT controls (**Figure 4.2C**). *Tcf-1*$^{-/-}$ DN1 cells had reduced levels of surface c-kit (**Figure 4.3B**) and an increased fraction of them expressed intermediate levels of CD25 (**Figure 4.2A and 4.2B**). In agreement with earlier reports, DN2 cells as well as all subsequent thymic subsets were dramatically reduced. Thus, Tcf-1 is required earlier than previously described, at, or prior to, thymic entry of uncommitted thymus-seeding progenitors (TSP). The reduction of ETP observed in *Tcf-1*$^{-/-}$ mice could reflect either a defect in migrating from the BM to the thymus or a cell-intrinsic inability of mutant TSP to develop towards the T-cell lineage. BM progenitors migrate to

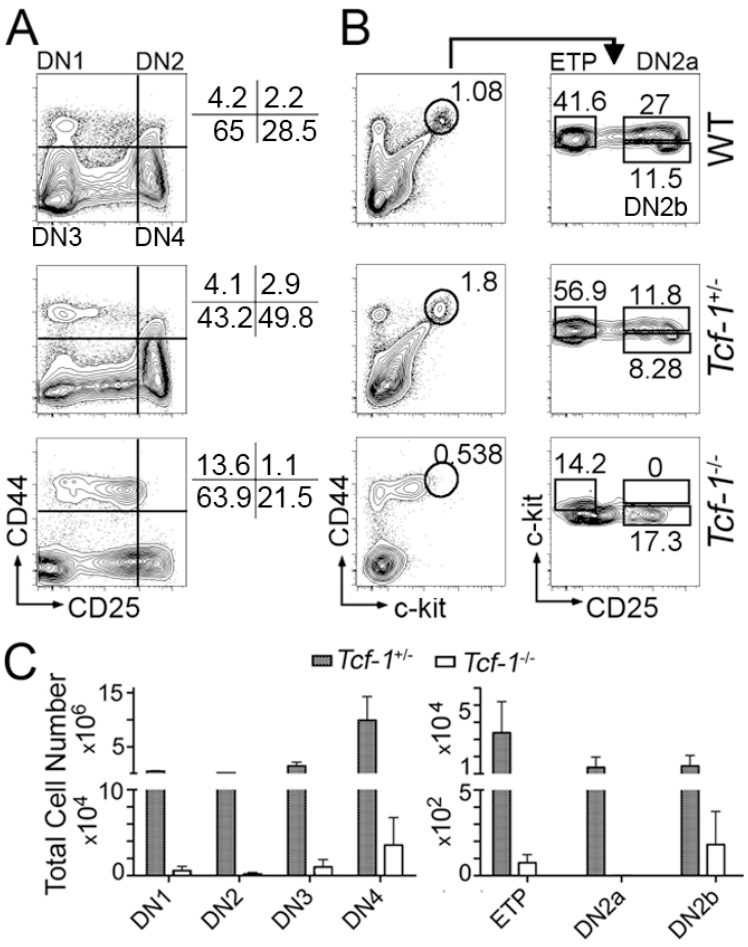

Figure 4.2: *Tcf-1*$^{-/-}$ mice have severely reduced ETPs and DN2 cells. Profiles of gated Tcf-1$^{-/-}$ and control Lin^{-} DN thymocytes are shown. **A**. CD44 versus CD25 profiles. The DN1-DN4 subsets are indicated. Numbers indicate the frequencies of each subset. **B.** CD44 versus c-kit profiles show the electronic gating for pro-T-cells (left). c-kit versus CD25 profiles of gated pro-T-cell cells define the ETP, DN2a and DN2b subsets as indicated (right). **C.** Bar-histograms of cell numbers in the indicated subsets of *Tcf-1*$^{-/-}$ and *Tcf-1*$^{+/-}$ mice. N= 4-6. (Germar et al., 2011)

the thymus via the bloodstream, which has been shown to contain progenitor subsets with T-lineage potential. These include blood LSK ($Lin^-c\text{-}kit^+Sca\text{-}1^+$), CLP ($Lin^-IL\text{-}7R\alpha^+Flt3^+c\text{-}kit^{lo}Sca\text{-}1^{lo}$) (Krueger and von Boehmer, 2007; Schwarz and Bhandoola, 2004), and the circulating-T-cell-progenitors (CTP) that express a human CD25 reporter of the pre-T-cell receptor a chain (HuCD25) (Krueger and von Boehmer, 2007). To examine whether *Tcf-1*$^{-/-}$ progenitors efficiently migrate through the bloodstream, we compared the presence of blood LSKs, CLPs and CTPs in *Tcf-1*$^{-/-}$ mice and littermate controls. LSKs and CLPs were present in the blood of *Tcf-1*$^{-/-}$ mice at frequencies comparable to control littermates (**Figure 4.4A** and **4.4B**). The presence of CTP was surveyed in the blood of *Tcf-1*$^{-/-}$ mice crossed to the transgenic HuCD25 reporter that was used for their identification (Krueger and von Boehmer, 2007). Like LSK and CLP, CTP were also present in the blood of *Tcf-1*$^{-/-}$ mice in frequencies comparable to control littermates (**Figure 4.4C**). Thus, *Tcf-1*$^{-/-}$ progenitors are able to migrate through the bloodstream further suggesting that the earliest defect is at or shortly after thymic entry.

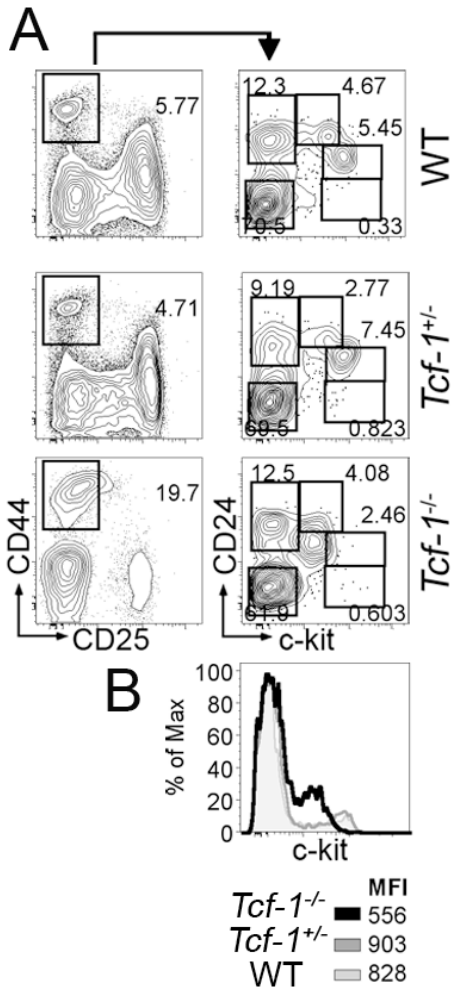

Figure 4.3: *Tcf-1*$^{-/-}$ ETP cells express low levels of c-kit. Profiles of gated Tcf-1$^{-/-}$ and control Lin^{-} DN thymocytes are shown. **A.** Analysis of the DN1 subsets in Tcf-1$^{-/-}$ and control mice. CD44 versus CD25 profiles show the gating of DN1 pro-thymocytes (left). CD24 versus c-kit profiles of gated DN1 cells define the DN1a (c-kit^{+}CD24^{-}), DN1b (c-kit^{+}CD24^{+}), DN1c (c-kit^{lo}CD24^{+}), DN1d (c-kit^{-}CD24^{+}), and DN1e (c-kit^{-}CD24^{-}) subsets. **B.** Histogram overlay of c-kit levels in gated DN1 thymocytes from the indicated mice. MFI=mean fluorescence intensity of c-kit.

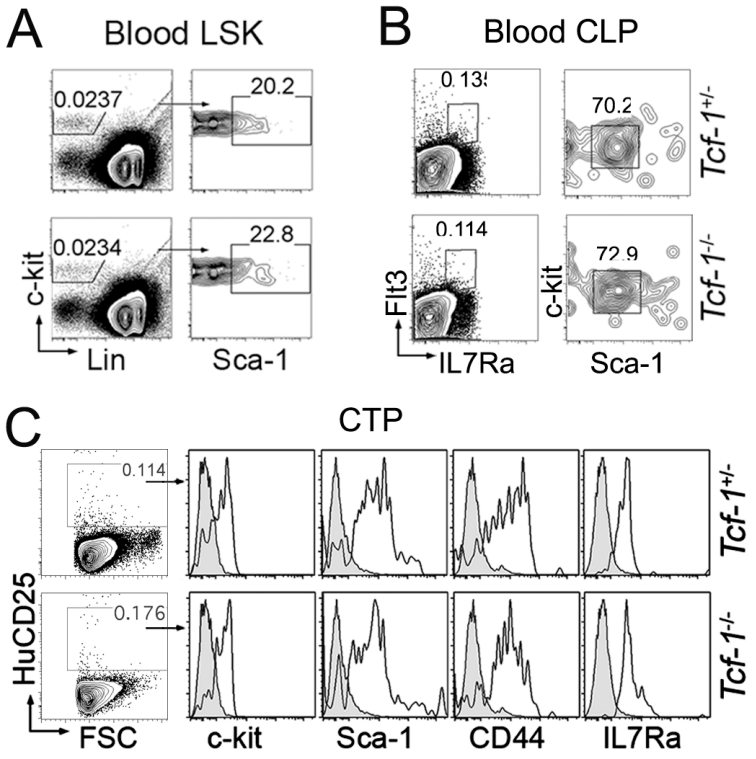

Figure 4.4: Circulating blood progenitors in *Tcf-1*$^{-/-}$ mice are normal in number. 0.6-1x10^{6} lymphocytes isolated from the blood of 6 mice for each group were stained for Lin as well as the indicated markers. **A.** Gating strategy for blood LSKs in the indicated mice. **B**. Gating strategy for blood CLPs. **C**. CTPs were gated as Lin^{-}, huCD25^{+}. Histogram overlays show the surface profile for markers that define the CTP in the indicated mice. Line histograms depict the CTPs and filled histograms depict an unstained negative control. Data are representative of 2 experiments. (Germar et al., 2011)

4.2 Notch1 can directly activate Tcf-1

Transcriptional upregulation of *Tcf7* started at the ETP stage (**Figure 3.1**) and coincided with the developmental block in *Tcf-1*$^{-/-}$ progenitors. The profile of early thymocytes in *Tcf-1*$^{-/-}$ mice resembled that of compound *Notch1*-deficient and *Notch2*-haploinsufficient (MxCre;*N1*$^{fl/fl}$*N2*$^{fl/+}$) thymocytes (**Figure 4.5**). Notch1 signaling in the ETP is a critical T-cell specification event. Notch1 deficiency or overexpression of Notch1 antagonists resulted in early arrest of T-cell development at the DN1 stage and an accumulation of B cells in the thymus (Feyerabend et al., 2009; Izon et al., 2002; Koch et al., 2001; Maillard et al., 2004; Radtke et al., 1999; Wilson et al., 2001). Despite the ETP block in *Tcf-1*$^{-/-}$, an increased distribution of B cells or macrophages was not observed in *Tcf-1*$^{-/-}$ thymi (**Figure 4.6**), indicating that Tcf-1 may not be involved in the suppression of B-cell fate.

Given the key role that Notch-1 plays in triggering the T-cell differentiation process, and considering the profound and early defects resulting from Tcf-1 ablation, we hypothesized that Notch-1 could directly activate *Tcf7*. Intriguingly, chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) of activated Notch-1 and CSL in the mouse T-cell lymphoma cell line T6E (Wang et al., 2011) revealed that both bind to an evolutionarily conserved CSL consensus site ~31.5 kb upstream of the *Tcf-1* transcription start site (**Figure 4.7**). ChIP-seq for Tcf-1 was performed in primary thymocytes of WT mice and also revealed strong Tcf-1 binding to an adjacent Tcf consensus site. The

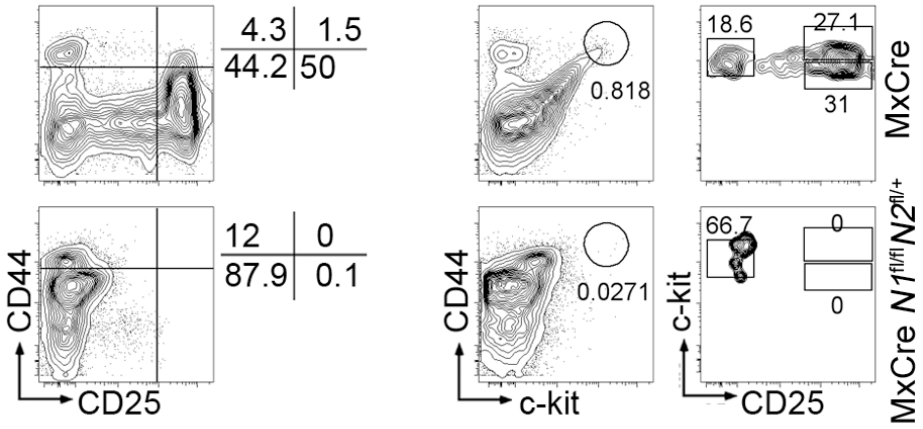

Figure 4.5: **Notch-deficient mice show a reduction in ETPs and a lack of DN2 cells.** Plots to the left show the CD44 versus CD25 profile of gated Lin^- thymocytes from MxCre and MxCre $Notch1^{fl/fl}$ $Notch2^{fl/+}$ (MxCre $N1^{fl/fl}N2^{fl/+}$) mice as indicated. Numbers to the right of the plots are quadrant frequencies. CD44 versus c-kit plots to the right show the electronic gating for pro-T-cells in the indicated mice. c-kit versus CD25 plots are gated pro-T-cell cells and depict the ETP, DN2a and DN2b subsets for the indicated mice. Numbers in the plots show gate frequencies. (Germar et al., 2011)

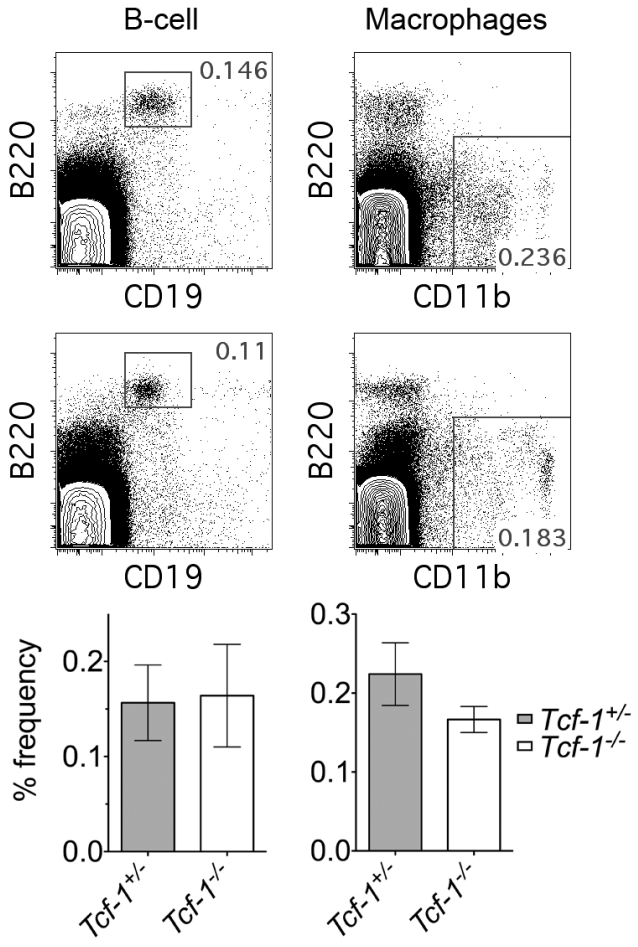

Figure 4.6: B cells and macrophages in *Tcf*-1$^{-/-}$ thymi are normal in ratio. Thymocyte suspensions from Tcf-1$^{-/-}$ or Tcf-1$^{+/-}$ controls were stained with antibodies to B220, CD19 and CD11b. Plots show B220 versus CD19 and B220 versus CD11b thymocyte profiles. Gates in B220 versus CD19 plots depict B cells, and gates in B220 versus CD11b plots depict macrophages. Histogram bars are average frequencies of B cells and macrophages as defined by the gates in the plots from 3 independent mice. Error bars are standard deviation. (Germar et al., 2011)

evolutionary conservation of this region and the active binding of Notch-1, CSL, and Tcf-1 to it suggest that it may represent an enhancer element.

To further confirm Notch binding to this sequence, we performed electromobility shift assay (EMSA) using oligonucleotides containing the conserved CSL motif. Nuclear extracts from the E2A-deficient 1F9 lymphoma cell line that has active Notch signaling (Engel and Murre, 2002) as well as purified CSL were probed for their ability to bind to this site. Mutant oligos in which the central 4 nucleotides of the CSL consensus binding site were converted to adenines (A) were designed as negative controls (see *Materials and Methods*). These analyses revealed specific binding of purified CSL to the oligo probe but not to the negative control (**Figure 4.7**, lanes 5-7). When incubated with nuclear extracts the oligo probe migrated slower than the complex with only purified CSL (**Figure 4.7B,** compare lanes 2 and 5). This slower migration of the protein DNA complex observed in 1F9 extracts suggests the presence of a multi-protein complex comprising factors such as Notch in addition to CSL at this site.

In a third approach to establish that Notch directly regulates Tcf-1, we examined the levels of *Tcf-1* expression in LSK BM progenitors ectopically expressing an activated, intracellular Notch (NotchIC). To this aim we interrogated existing microarray data (**GSE27799** (Klinakis et al., 2011) and compared the expression profiles of LSK BM progenitors sorted before or after MxCre mediated induction of a dormant oncogenic Notch1-IC (Buonamici et al., 2009). Indeed, *Tcf-1* was sharply upregulated by more than 10-fold in response to Notch1-IC induction, paralleling the behavior of a panel of known Notch targets

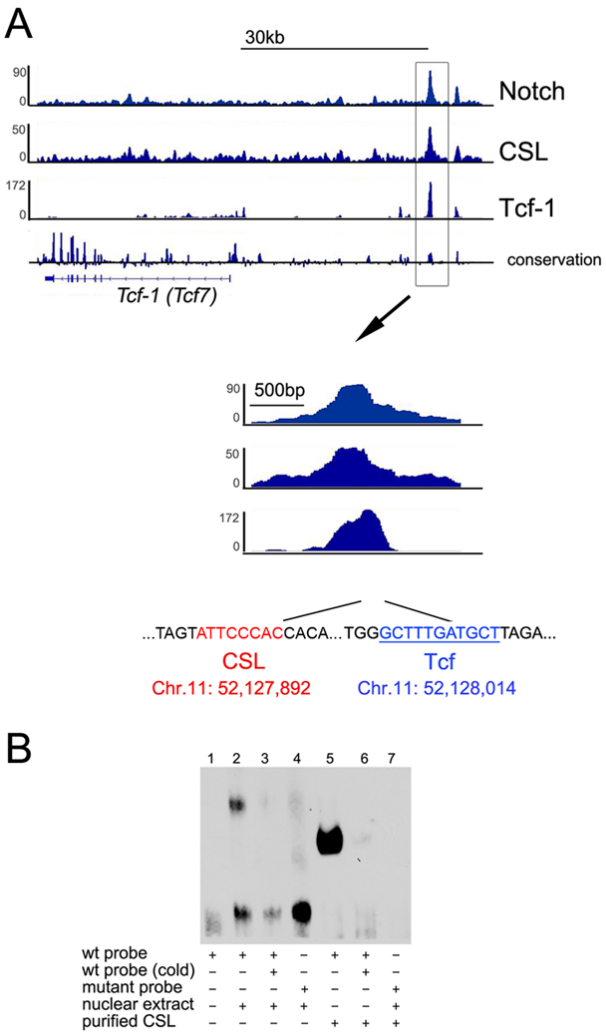

Figure 4.7: Tcf-1 is a direct target of Notch1. A. Visualization of ChIP-seq data in the *Tcf-1 (Tcf7)* locus. Notch-1 and CSL ChIP-seq data are from the T6E cell line, Tcf-1 ChIP-seq data are from WT thymocytes. Mammalian conservation is shown under the data tracks. Magnification of the indicated area strongly bound by Notch-1, CSL, and Tcf-1 is shown (ca. 31.5kb upstream of the *Tcf-1* TSS). This region contains conserved CSL and Tcf binding sites as indicated. *Notch1 ChIP-seq performed by Hongfang Wang*. **B**. EMSA with nuclear extracts from the 1F9 T-ALL cell line (lanes 2-4) or purified CSL (lanes 5-7). Probes are from the conserved CSL binding site in the putative Tcf-1 enhancer region, starting at position 52127891 on chromosome 11 (mm9). (Germar et al., 2011)

(**Figure 4.8**). In conclusion, the temporal regulation of *Tcf-1* expression, the ChIP-seq and EMSA data, as well as the upregulation of *Tcf-1* in response to ectopic Notch1-IC activation show that *Tcf-1* is directly regulated by Notch.

4.3 Tcf-1 is specifically required at the c-kit^{+} DN1 cell subset.

The earliest defect observed in *Tcf-1*$^{-/-}$ thymic development was the reduced level of c-kit expression at the DN1 stage and the presence of an increased fraction of CD25^{int} CD44^{+} DN thymocytes (**Figure 4.2**). Similar thymic phenotypes have been observed in mice deficient for transcription factors implicated in early T-cell differentiation, including *Gfi1* (Yucel et al., 2003). To gain insights into the properties of the c-kit^{lo} DN1 *Tcf-1*$^{-/-}$ thymocytes we compared them to the c-kit expressing DN1 thymocytes in control mice. *Tcf-1*$^{-/-}$ thymi had a 30-fold reduction in c-kit^{lo} DN1 cells as compared to controls. While these cells proliferated at similar rates to their control counterparts, they were more apoptotic (**Figure 4.9A**). We further determined the molecular impact of Tcf-1 ablation in these cells by comparing their global expression profiles to that of c-kit^{+} DN1 cells from *Tcf-1*$^{+/-}$ controls. To this aim, RNA was prepared from Lin^{-} CD44^{+}CD25^{-}kit^{+} cells (**Figure 4.9A**) and profiled on microarrays. Gene ontology (GO) analysis revealed an overall enrichment in the expression of genes involved in hemopoiesis and immune system development (**Figure 4.9B**). While genes involved in T-cell specification including *Notch1, Hes1, Gata3, Bcl11b, Runx1, Ikzf1* (*Ikaros*) were largely unchanged in *Tcf-1*$^{-/-}$ cells (**Figure 4.9C**), expression

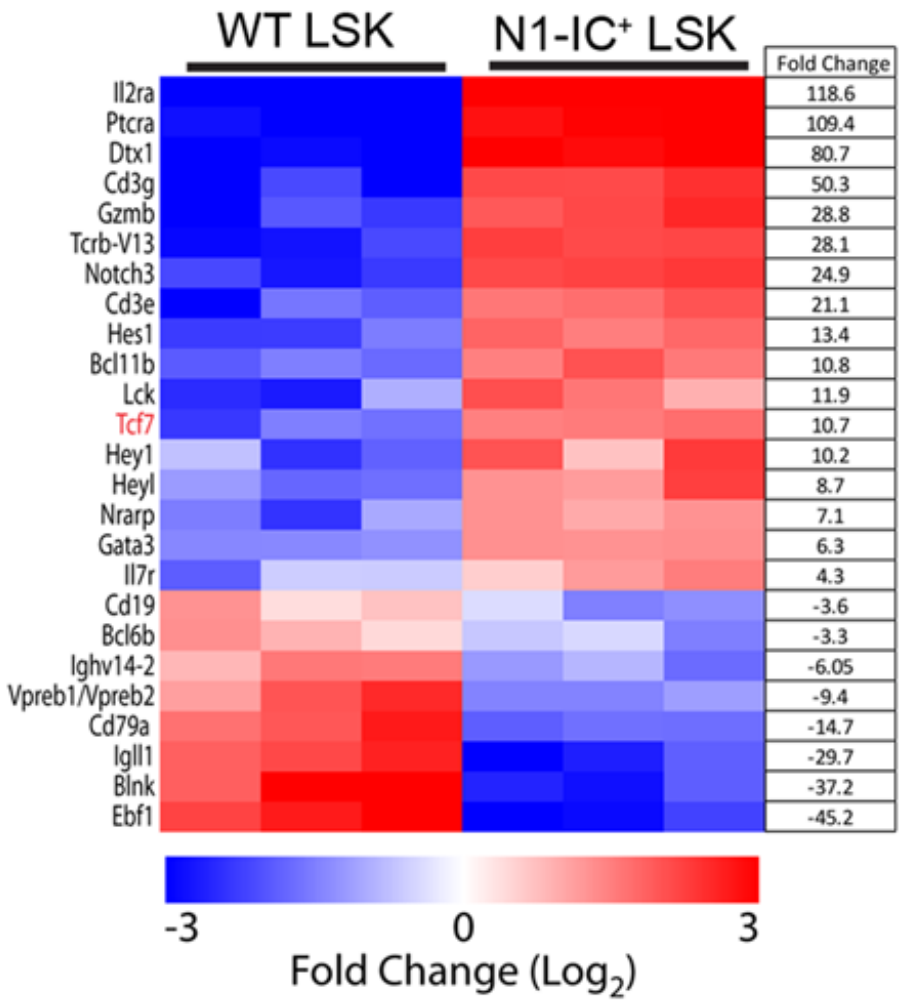

Figure 4.8: Tcf-1 upregulation in response to Notch1 induction. Heat map depicting the expression of the indicated genes in LSK BM progenitors sorted before or after MxCre mediated induction of a dormant oncogenic Notch1-IC (N1-IC$^+$). Fold change of expression between N1-IC$^+$ and WT LSK samples. *Microarray performed by Camille Lobry*. (Germar et al., 2011)

of some genes such as *Id2, Tcf3* (E2A) and *Il7r* was elevated. Most T-cell signature genes showed unchanged expression (*Ptcra, Lck, Zap70, Lat)*, but *Cd3d* and *Cd3e* were down- and *Cd4* was upregulated. Interestingly, these analyses indicate a dramatic overall downregulation of genes associated with response to DNA damage stimuli, chromatin remodeling, and DNA metabolic processes in the Tcf-1 deficient progenitors. This is consistent with our observation that these cells also show increased cell death. Our findings therefore indicate that Tcf-1 is required to maintain basic metabolic processes in early thymic immigrants, which, in its absence, become abortive and die by apoptosis.

4.4 Tcf-1 functions independently of the canonical Wnt signaling pathway.

The normal distribution of BM and blood progenitors compared to the reduced presence of *Tcf-1*$^{-/-}$ ETPs, and the deregulation of gene expression patterns in these cells suggested a functional inability to progress along the T-cell lineage. To address this possibility we examined the T-cell potential of hematopoietic progenitors from *Tcf-1*$^{-/-}$ mice in co-cultures on OP9-DL1 stroma cells. These cells instruct differentiation to the T-cell lineage because they express Delta-like-1 ligand to stimulate Notch signaling. HSC, MPP, LMPP, and CLP were isolated from *Tcf-1*$^{-/-}$ (CD45.2^{+}) and WT (CD45.1^{+}) mice. *Tcf-1*$^{-/-}$ and WT sorted subsets were mixed at a 1:1 ratio and co-cultured on OP9-DL1 stroma (**Figure 4.10A**). *Tcf-1*$^{-/-}$ cells failed to upregulate CD25 and progress

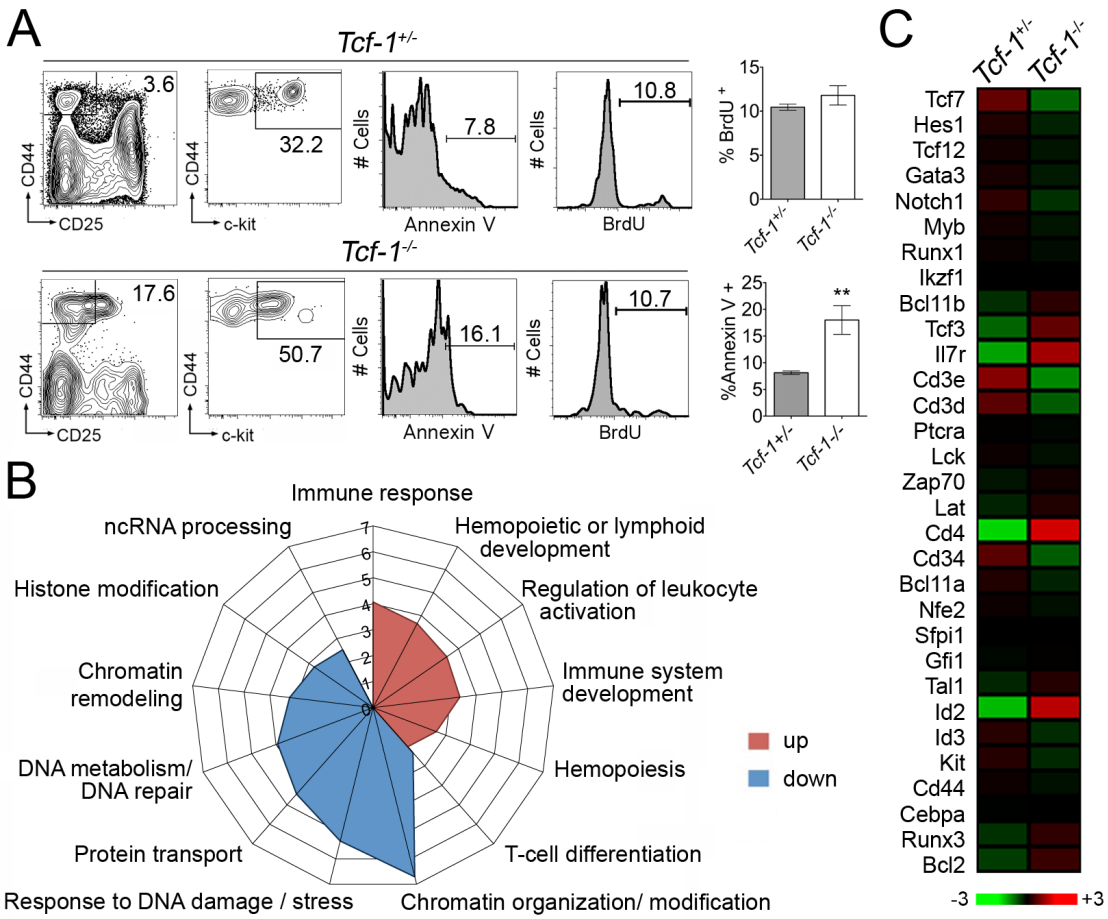

Figure 4.9: Comparison of the c-kit+ DN1 thymocytes subset in *Tcf-1*$^{-/-}$ versus WT mice. A. CD44 versus CD25 plots depict the gating of DN1 cells in *Tcf-1*$^{+/-}$ versus *Tcf-1*$^{-/-}$ mice. CD44 versus c-kit plots are DN1 cells from *Tcf-1*$^{+/-}$ and *Tcf-1*$^{-/-}$ mice as indicated. The gate in these plots depicts Lin^{-}CD44^{+}c-kit^{+}CD25^{-} (ETP) cells analyzed for apoptosis proliferation and global gene expression. Annexin^{+} and BrdU histograms depict the fraction of apoptotic and proliferating ETPs, respectively, in the indicated mice (*Materials and Methods*). **B.** GO analysis of up- and down- regulated genes in the indicated processes in *Tcf-1*$^{-/-}$ versus *Tcf-1*$^{+/-}$ ETPs. Scale shows fold enrichment. **C.** Heat map showing the mean expression of select genes representative of the ETP/T-cell signature from the indicated mice. Means are the average of three biological replicates. (Germar et al., 2011)

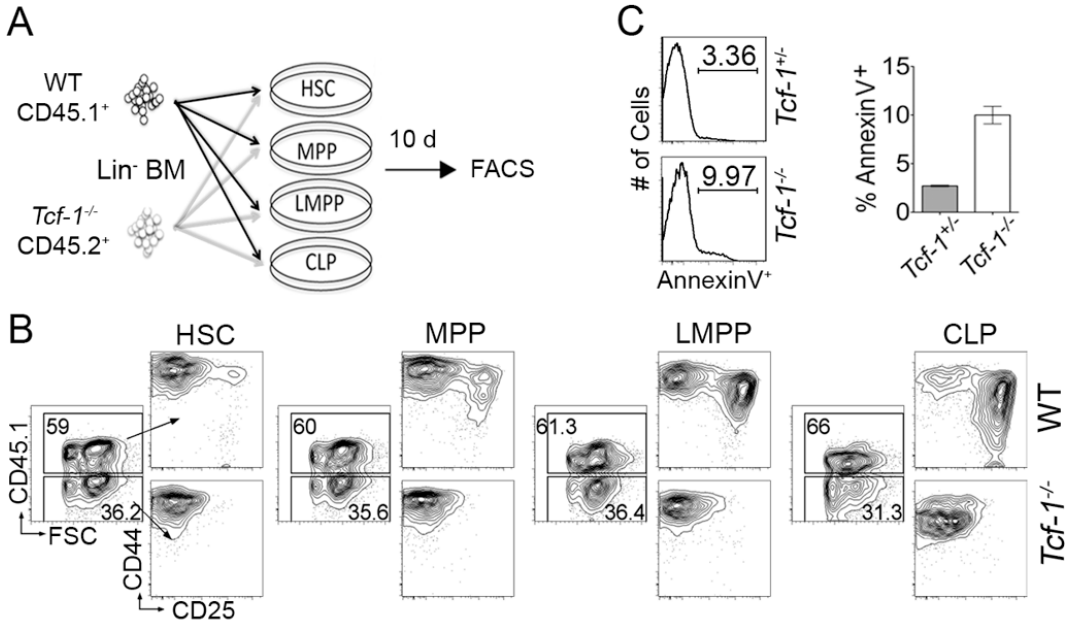

Figure 4.10: *Tcf-1*$^{-/-}$ progenitors show defective T-cell potential in OP9-DL1 cocultures. A. Experimental scheme. Lin^{-} BM precursors or progenitor subsets, were sorted from *Tcf-1*$^{-/-}$ or WT mice. Equal numbers of *Tcf-1*$^{-/-}$ or WT progenitors were mixed and co-cultured on OP9-DL1 stroma for 10 days. **B.** OP9-DL1 cultures of the indicated progenitors. CD45.1 versus FSC plots define the gating of *Tcf-1*$^{-/-}$ (CD45.1^{-}) versus WT (CD45.1^{+}) cells. CD44 versus CD25 plots of the gated cells as indicated by arrows. Similar results were obtained in three independent experiments. **C.** Apoptosis of *Tcf-1*$^{-/-}$ and control progenitors in OP9-DL1 cultures was measured by Annexin V staining. Histograms show Annexin V staining of control (upper) and *Tcf-1*$^{-/-}$ (lower) Lin^{-} BM progenitors cultured on OP9-DL1 co-cultures for 10 days. Histogram bars show the average frequency of Annexin V+ cells from the indicated mice in 3 co-cultures. Error bars are standard deviation. (Germar et al., 2011)

towards the T-cell lineage (**Figure 4.10B**). Additional experiments revealed that such non-differentiating *Tcf-1*$^{-/-}$ progenitors contained a larger fraction of early apoptotic (AnnexinV^{+}) cells compared to their control (*Tcf-1*$^{+/-}$) counterparts (**Figure 4.10C**).

Remembering that Tcf-1 can function as either a transcriptional activator or repressor depending on its binding to β-catenin or Groucho, respectively, another question that remains in our understanding of the regulation of Tcf-1 transcriptional activity is whether the defects observed in Tcf-1-deficient mice are dependent on β-catenin. To address this, *Tcf-1*$^{-/-}$ progenitors were retrovirally transduced to express either wildtype or mutant Tcf-1 containing a deletion in the β-catenin-binding domain (ΔβCBD). Transduced progenitors were then cultured on OP9-DL1 cells and assessed for development toward the T-lineage by FACS staining of CD44 and CD25 expression. As expected, transduction of wildtype Tcf-1 into Tcf-1-deficient progenitors rescued their ability to develop toward the T-lineage as demonstrated by the upregulation of CD25 on c-kit^{+} cells (**Figure 4.11**, middle panel). Interestingly, transduction of *Tcf-1*$^{-/-}$ progenitors with Tcf-1 ΔβCBD was also able to rescue the T-cell defect (**Figure 4.11**, bottom panel). Our evidence suggests that Tcf-1 has a functional role in T-cell development that does not depend on its binding to β-catenin.

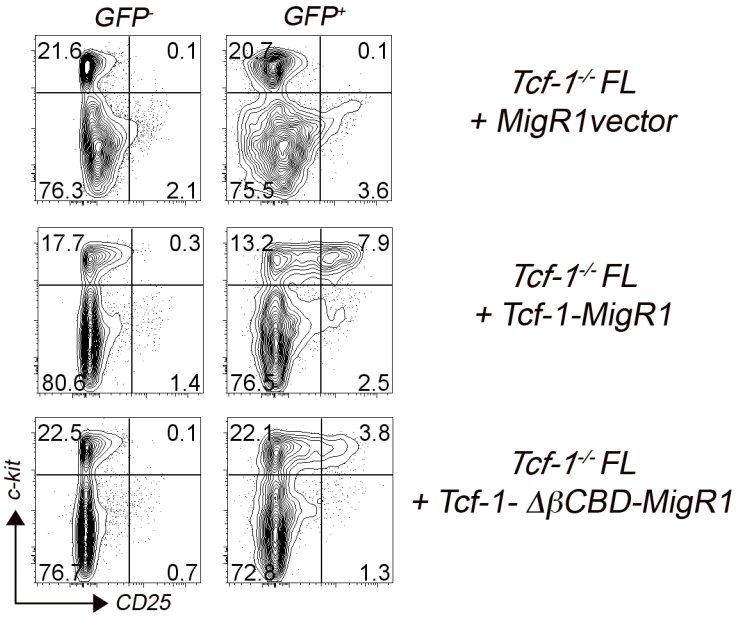

Figure 4.11: Binding to β-catening is dispensable for early development of T-cell progenitors on stromal cell culture. Virus containg empty vector, full length Tcf-1, or Tcf-1 with a deletion of the β-catenin binding domain (BD) (aa 1-69) were was used to infect Lin depleted, c-kit^{+} fetal liver cells from *Tcf-1$^{-/-}$* mice. Transduced Tcf-1-progenitors were then cultured onto OP9-DL1 stromal cells. Infected *Tcf-1$^{-/-}$* cells were detected by the presence of GFP, and their development was assessed on day 10 of stromal cell co-culture for the presence of c-kit and CD25. Profiles of c-kit versus CD25 are shown for *Tcf-1$^{-/-}$* cells infected with the vectors indicated. N=3 for each condition.

4.5 *Tcf-1*$^{-/-}$ progenitors are cell-intrinsically defective in T-cell specification.

To establish that Tcf-1 deficiency did not interfere with thymic entry we generated competitive BM chimeras using Lin$^-$ BM progenitors from *Tcf-1*$^{-/-}$ and WT mice. Equal numbers of *Tcf-1*$^{-/-}$ progenitors were combined with WT progenitors and injected intravenously to reconstitute lethally-irradiated congenic mice (**Figure 4.12A**). Ten weeks after adoptive transfer BM, thymus, and spleen were analyzed to compare the relative contribution of *Tcf-1*$^{-/-}$ and WT progenitors to the various stages and lineages of hematopoietic development. *Tcf-1*$^{-/-}$ cells efficiently reconstituted BM progenitor subsets and other mature blood lineages as determined by their frequency among B cells (B220$^+$) and DC (CD11c$^+$) (**Figure 4.12B**). The distribution of BM progenitors, mature B cells, DCs, and macrophages appeared to be unaffected by loss of Tcf-1 (**Figures 4.12B** and **4.13**). This was expected as analysis of the *Tcf-1*$^{-/-}$ mice also revealed a normal distribution B220$^+$ and CD11b$^+$ cells (**Figure 4.14**). By contrast, NK1.1$^+$ cells from *Tcf-1*$^{-/-}$ donors were outcompeted in the spleen (**Figure 4.12**), and our follow-up analysis is discussed in the next section. Interestingly, within the thymus of the chimeras, *Tcf-1*$^{-/-}$ progenitors did repopulate the ETP stage (**Figure 4.12C**). Their fraction in this subset was reduced compared to BM progenitors, however this reduction was not statistically significant, indicating that Tcf-1 requirement here is not completely cell intrinsic. *Tcf-1*$^{-/-}$ progenitors were strikingly outcompeted in all subsequent stages of intrathymic and peripheral T-cell development (**Figures 4.12D** and **4.12E**). Altogether, our findings *in vivo* and *in vitro* allow the

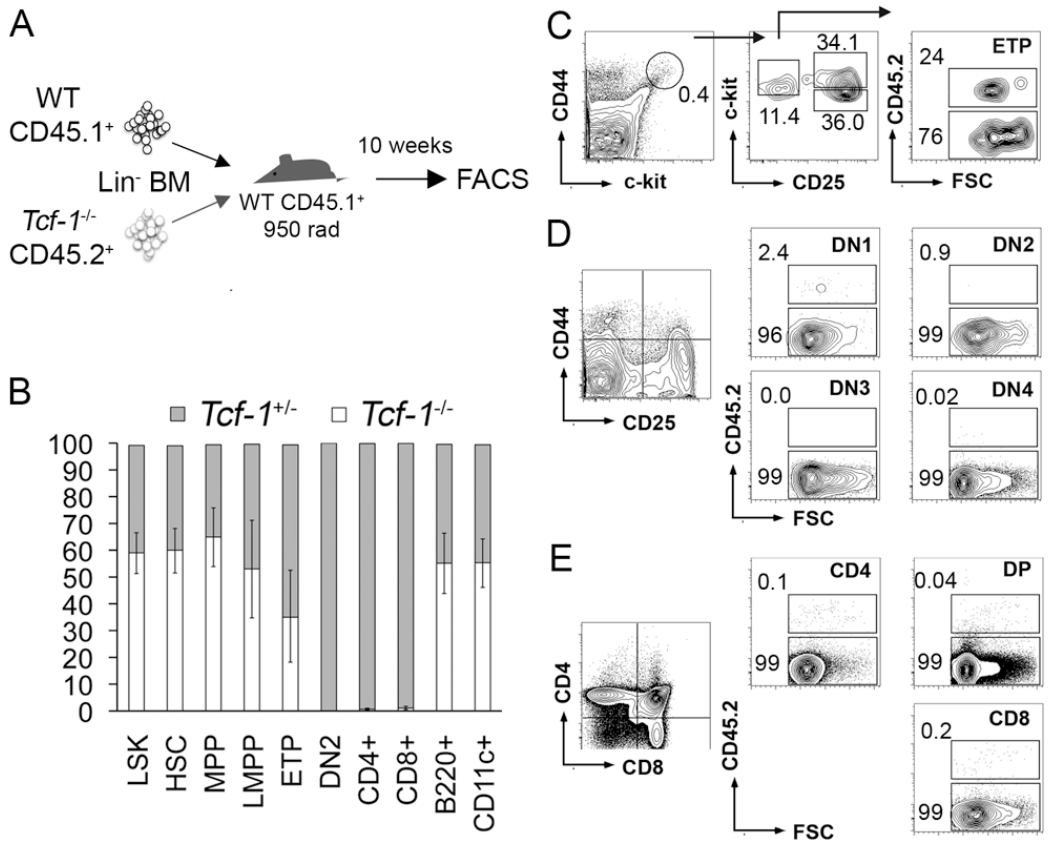

Figure 4.12: *Tcf-1*$^{-/-}$ progenitors are selectively defective in T-cell development. A. Experimental scheme. 5x10^5 CD45.2$^+$ Lin$^-$ BM progenitors sorted from *Tcf-1*$^{-/-}$ were mixed with an equal number of WT CD45.1$^+$ Lin$^-$ BM progenitors and injected intravenously into lethally irradiated mice. After 10 weeks cells were harvested from BM, spleen and thymus, and analyzed. Progenitor subsets and mature lineages were gated as indicated to determine the fraction of CD45.2$^+$ *Tcf-1*$^{-/-}$ cells versus CD45.2$^-$ WT cells in each population. (**B**) Histogram bars depict the relative contribution of *Tcf-1*$^{-/-}$ versus WT progenitors in reconstituting the indicated progenitor subsets and mature lineages. Values represent the average of five independent mice. (**C-E**) Gating strategy for ETP (**C**), DN1-DN4 (**D**), DP and SP subsets (**E**). Similar results were obtained with more than 10 reconstituted mice in three independent experiments. (Germar et al., 2011)

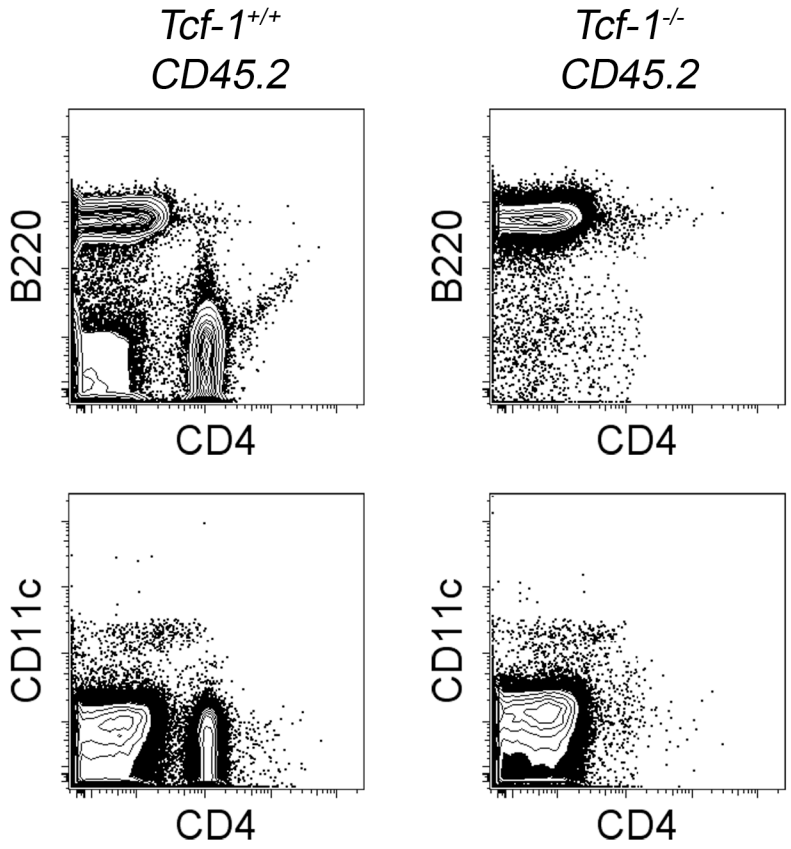

Figure 4.13: Tcf-1$^{-/-}$ progenitors give rise to B cells and CD11c^{+} cells in competitive mixed BM chimeras. BM progenitors from Tcf-1$^{-/-}$ (CD45.2) mice were isolated by sorting and mixed with their WT CD45.1 counterparts before injecting WT lethally irradiated (CD45.1) recipients. 10 weeks after injection splenocytes were stained with the indicated surface markers and analyzed. (Upper) B220 versus CD4 are the profiles of gated *Tcf-1$^{-/-}$* (CD45.2^{+}) or *Tcf-1$^{+/+}$* (CD45.2^{-}) cells isolated from the recipients. (Lower) CD11c versus CD4 are the profiles of gated *Tcf-1$^{-/-}$* (CD45.2^{+}) or *Tcf-1$^{+/+}$* (CD45.2^{-}) cells isolated from the recipients. (Germar et al., 2011)

conclusion that *Tcf-1*$^{-/-}$ uncommitted progenitors can efficiently migrate through the bloodstream and enter the thymus, however they are cell-intrinsically defective in T-cell commitment.

In conclusion, the detailed analysis of early thymocyte subsets in Tcf-1 deficient mice yielded a novel picture of the hierarchy of transcription factor governing early thymocyte development. Our studies identified Tcf-1 as a direct target of Notch that is necessary for development to the T-cell fate. Interestingly, this function appeared to be independent of binding to β-catenin. Additionally, analyses of mixed BM chimera experiments indicated that the effects of Tcf-1 deficiency might extend beyond the T-cell compartment to the NK lineage. This led us to further investigation of possible roles for Tcf-1 in NK cell development, presented in the next chapter.

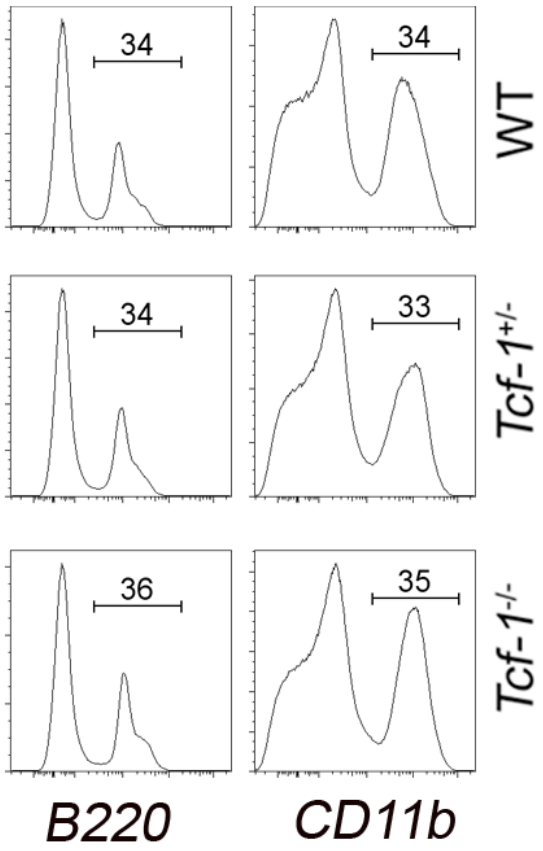

Figure 4.14: Tcf-1$^{-/-}$ mice have a normal distribution of B cells and CD11c^{+}. Analysis of BM cells for B220 (left) and CD11b (right). Representative of 3 mice.

CHAPTER V:

RESULTS III: TCF-1 IN NK-CELL DEVELOPMENT

Having established the earliest requirement for Tcf-1 in T-cell development, we were also interested in investigating the effect of Tcf-1 deficiency on other cells of the hematopoietic compartment. As mentioned, we observed no defects in the generation of B cells and DCs. This is in alignment with the absence of Tcf-1 expression in these cells. In contrast, we observed that splenic NK1.1^{+} cells from *Tcf-1*$^{-/-}$ donors were outcompeted in mixed BM chimeras (see *Chapter IV*). Tcf-1 expression has been detected in both human and murine NK cells, and Tcf-1 has been reported to influence the Ly49 repertoire through direct binding of regulatory elements in Ly49 receptor genes (Held et al., 1999; Ioannidis et al., 2003; Kunz and Held, 2001; Toor et al., 2001). While several factors have been implicated in the development of mature NK cells and their acquisition of effector function, only a few have been identified to play a role in the developing NK cells. Moreover, whether these factors might contribute to the specification of the NK lineage in the BM is unknown. Thus, it was of great interest to us to investigate NK cell development in the absence of Tcf-1.

5.1 Tcf-1 is expressed in NK cells

Since clonal acquisition of the diverse members of the Ly49 family begins at the iNK stage, and since Tcf-1 has been implicated in the direct regulation of Ly49A and potentially other Ly49 receptors in peripheral NK cells, we wanted to investigate whether Tcf-1 could also be important in the ontogeny of BM NK

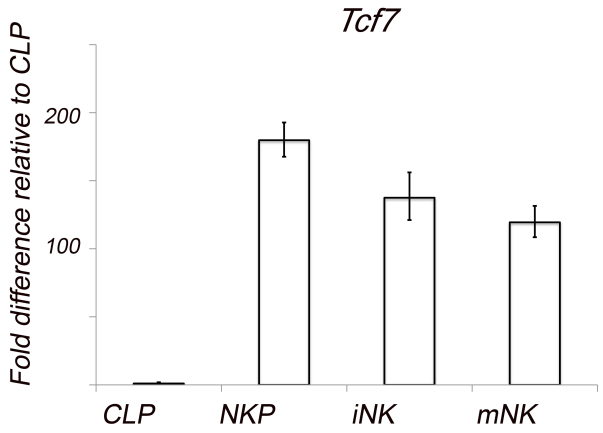

Figure 5.1: ***Tcf7* expression in BM NK precursors.** CLP, NKP, iNK, and mNK as defined in Figure 1.3 were sorted from the BM of WT mice. RNA was extracted and analyzed by qPCR. Tcf-1 expression was normalized to GAPDH. Histograms show relative expression levels in the various subsets normalized to the levels of CLP. Error bars represent standard deviation from technical triplicates. Data are representative of two experiments.

cells. While *Tcf7* expression has been found in mature NK cells, *Tcf7* expression has not yet been reported in the BM compartment. We first sought to establish whether *Tcf7* is expressed in developing NK cells. As with the BM progenitors we have examined, NKP ($Lin^-IL\text{-}2R\beta^+NK1.1^-DX5^-$), iNK ($Lin^-IL\text{-}2R\beta^+NK1.1^+DX5^-$), and mNK ($Lin^-IL\text{-}2R\beta^+NK1.1^+DX5^+$) cells were isolated from BM, and *Tcf7* expression was measured by qPCR. Indeed, *Tcf7* was expressed in NKP and cells of the subsequent iNK and mNK stages (**Figure 5.1**). Expression sharply increased from the earlier CLP progenitor stage, where *Tcf7* was barely detectable (**Figures 5.1** and **3.1**). Transcript level remained similar throughout the developing NK stages, though it trended toward a modest decrease after the NKP. *Tcf7* expression in NKP cells was not as high as in the ETP.

5.2 Tcf-1 is required for NK-cell development starting at the NKP stage.

Since we detected *Tcf7* transcript throughout the BM NK compartment, we hypothesized that loss of Tcf-1 would result in defective NK cell development. We reasoned that although the CLP was found to be unaffected in *Tcf-1*$^{-/-}$ mice, the distribution of the surface antigen Ly6D on the CLP might be affected. Ly6D demarcates the bifurcation of a progenitor with B potential from a progenitor with T and NK bi-potential, and we have already established profound defects in the T-cell compartment. Flow cytometric analysis of Ly6D distribution on CLP cells showed that the bipotent T/NK CLP was unaffected by Tcf-1 deficiency (**Figure 5.2**).

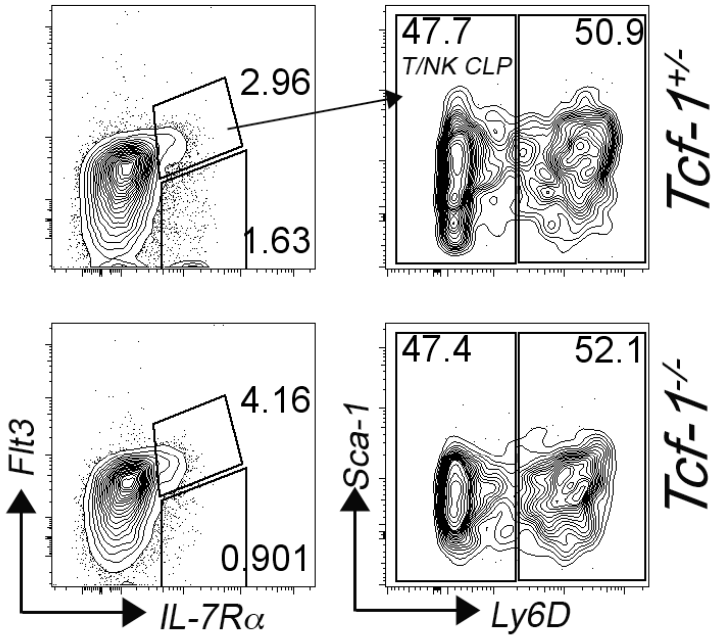

Figure 5.2: **Common T/NK progenitors in the BM are normal in number in *Tcf-1*$^{-/-}$ BM.** Analysis of gated Lin^{-} BM cells is shown. Flt3 versus IL-7Rα profiles show the gating for CLPs (left panel). Sca-1 versus Ly6D profiles on gated CLPs are shown in the right panel. Ly6D^{+} CLPs give rise to B cells while Ly6D$^{-/-}$ CLPs are precursors to T and NK cells.

Next, we examined the distribution and cellularity of NKP, iNK, and mNK within the BM. These analyses showed that *Tcf-1*$^{-/-}$ BM had about a 4-to-5-fold reduction in the ratio and absolute number of total Lin^{-}IL-2Rβ^{+} compared to *Tcf-1*$^{+/-}$ littermate controls (**Figure 5.3**). While pNK cells were significantly reduced in *Tcf-1*-deficient mice, iNK cells were almost absent, and mNK cells maintained a large 4-to-5-fold reduction.

5.3 Tcf-1$^{-/-}$ progenitors are cell-intrinsically defective in NK-cell specification.

The normal distribution of BM progenitors preceding the committed NKP compared to the reduction of *Tcf-1*$^{-/-}$ IL-2Rβ^{+} NK precursors suggests that Tcf-deficiency confers incompetency to progress along the NK cell lineage. Since NK cell development also depends on a number of cell extrinsic signals provided by the BM microenvironment, we wanted to address whether Tcf-1 deficient hematopoietic progenitors had a cell-intrinsic defect in NK-cell potential. To do this we generated competitive BM chimeras using Lin^{-} BM progenitors from *Tcf-1*$^{-/-}$ and WT mice. As in our examination of T-cell potential in *Tcf-1*$^{-/-}$ progenitors, equal numbers Tcf-1 deficient precursors were combined with WT progenitors and injected retro-orbitally to reconstitute lethally-irradiated congenic mice (**Figure 5.4**). Ten weeks after adoptive transfer BM, thymus and spleen were analyzed to compare the relative contribution of *Tcf-1*$^{-/-}$ and WT progenitors to

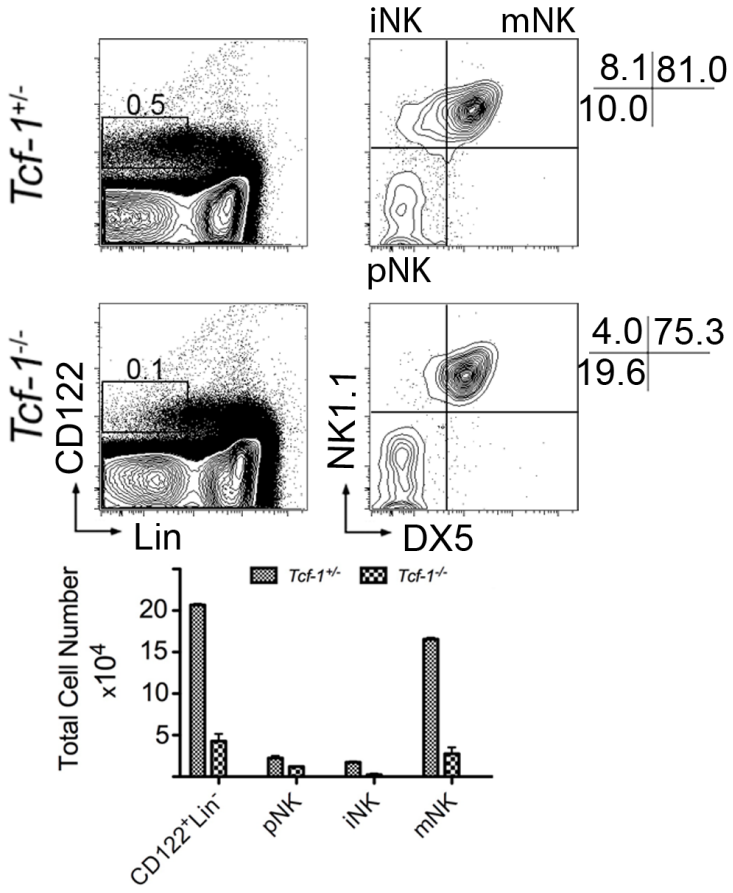

Figure 5.3: Tcf-1$^{-/-}$ mice have reduced NK progenitors. Profiles of gated Tcf-1$^{-/-}$ and control BM cells are shown. Panels on the left show the gating of CD122^{+}Lin^{-} cells. NK1.1 versus DX5 profiles (right) of gated CD122^{+}Lin^{-} cells define the pNK (NK1.1^{-}DX5^{-}), iNK (NK1.1^{+}DX5^{-}), and mNK (NK1.1^{+}DX5^{+}). Bar-histograms of cell numbers in the indicated subsets of Tcf-1$^{-/-}$ and Tcf-1$^{+/-}$ mice (N= 4-6) are depicted (bottom).

the various stages and lineages of hematopoietic development. *Tcf-1*$^{-/-}$ progenitors were competent in their ability to reconstitute the BM progenitor compartment but were outcompeted in all IL-2Rβ^{+} stages of BM NK stages of development, including the NKP, iNK, and mNK stages (**Figure 5.4**). This data indicates a cell-intrinsic defect of *Tcf-1*$^{-/-}$ progenitors to give rise to progeny of the NK lineage.

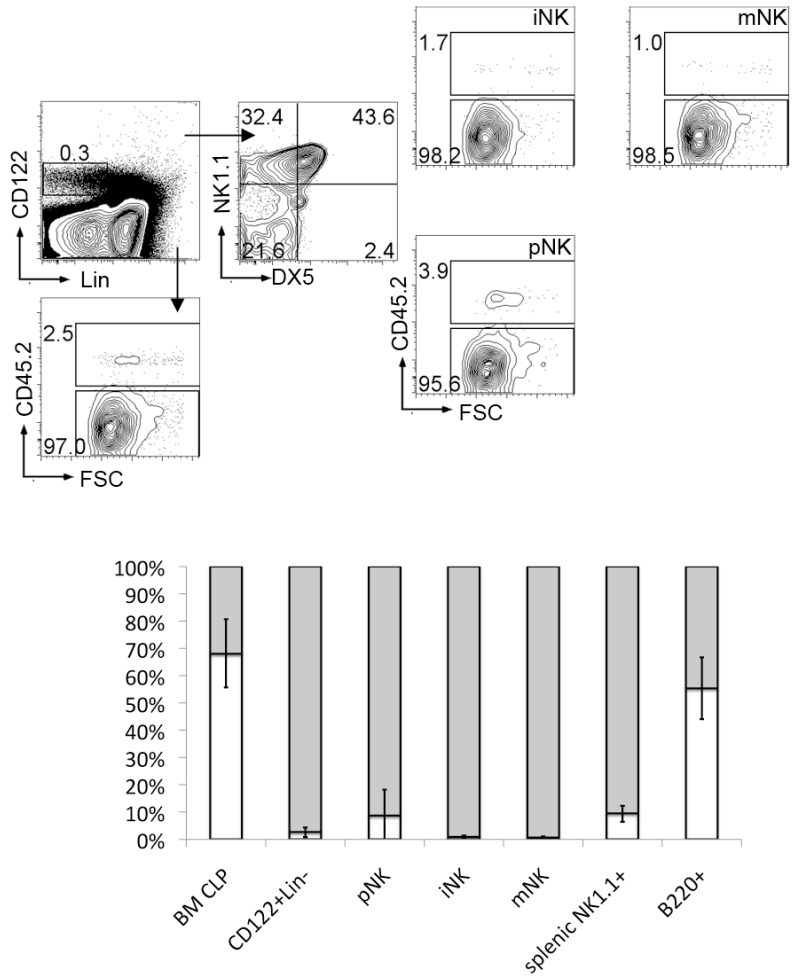

Figure 5.4: *Tcf-1*$^{-/-}$ progenitors are cell-intrinsically defective in NK-cell development. 5x10^5 CD45.2$^+$ Lin$^-$ BM progenitors sorted from *Tcf-1*$^{-/-}$ were mixed with an equal number of WT CD45.1$^+$ Lin$^-$ BM progenitors and injected intravenously into lethally irradiated mice (9.5 Gy). After 10 weeks cells were harvested from BM and spleen. Gating strategy for NK progenitors shown (top left). Progenitor subsets and mature lineages were gated as indicated to determine the fraction of CD45.2$^+$ *Tcf-1*$^{-/-}$ cells versus CD45.2$^-$ WT cells in each population. Histogram depicts the relative contribution of *Tcf-1*$^{-/-}$ (white bars) versus WT progenitors (gray bars) in reconstituting the indicated progenitor subsets and mature lineages (bottom). Values represent the average of five independent mice. Similar results were obtained with more than 10 reconstituted mice in three independent experiments.

CHAPTER VI:

DISCUSSION AND PERSPECTIVES

Roles for Tcf-1 have been described at the DN2 and the ISP stages of thymic development. Since these initial studies, our picture of early lymphoid development has gained greater definition. We set out to establish the beginning of Tcf-1 requirement in the lymphoid system and establish a place for Tcf-1 within the landscape of transcription factors known to govern T-cell development. While Tcf-1 is expressed in the HSC, the present study defines Tcf-1 as a gatekeeper of the earliest phase of T-cell specification, when Notch signals initiate the T-lineage program in uncommitted thymic progenitors. Tcf-1 is upregulated upon entry of seeding progenitors to the thymus, and expression is maintained throughout thymic development. We show here that transcriptional upregulation of Tcf-1 is essential to enable progenitors to progress beyond the ETP stage, and we provide evidence that the expression of factors involved in basic processes of DNA metabolism and chromatin organization is compromised, offering an explanation for why *Tcf-1*$^{-/-}$ ETPs are more apoptotic than control counterparts. Importantly, the expression of most T-cell specification factors remains unaltered. We show that Notch1 directly mediates *Tcf7* transcription. Finally, we demonstrate a novel role for Tcf-1 in early NK cell development.

6.1 Tcf-1 is expressed in early BM progenitors but is dispensable for their development toward the lymphoid lineages up to the CLP.

The Wnt signaling pathway has been implicated in the maintenance of HSCs, although its precise role has been controversial with gain- and loss-of

function studies reporting conflicting results. Since signaling components of the Wnt pathway are complexly regulated as well as play various Wnt-independent roles, we reasoned that the most direct way of investigating a requirement for Wnt in hematopoiesis, and lymphopoiesis more specifically, would be via the analysis of a mouse deficient in Tcf-1, the most downstream effector in the activation of Wnt-responsive genes. Here we report that while expression of *Tcf7*, the gene encoding Tcf-1, can be detected as early as the HSC, and while low levels of Tcf/Lef activity can be observed in BM progenitors (**Figure 3.1**), surprisingly, ablation of Tcf-1 did not affect the distribution, cellularity, or proliferation of HSCs or the subsequent MPP, LMPP, or CLP stages of hematopoietic development (**Figures 3.2** and **3.3**).

One explanation for the lack of an obvious phenotype of *Tcf-1*$^{-/-}$ BM progenitors despite its expression in the early HSC compartment could be that other members of the Tcf/Lef family may play a compensatory role in the absence of Tcf-1. Due to identical HMG-binding domains and high homology throughout the protein, Tcf-1 and Lef-1 have been suggested to play redundant roles in lymphopoiesis. Indeed, ablation of Lef-1 in combination in the hypomorphic *Tcf-1*$^{\Delta V/\Delta V}$ mice resulted in exacerbated thymic defects when compared to the hypomorph alone (Held et al., 2003). However, our analyses found no support for this hypothesis. While *Lef1* was expressed in CLP, in agreement with its function in B-cell development, transcripts of other Tcf/Lef family members were below the detection limit in wildtype BM progenitors. Loss of Tcf-1 did not change this pattern, and *Tcf7l1* (Tcf-3), *Tcf7l2* (Tcf-4), and *Lef1*

transcript levels were not upregulated in Tcf-1-deficient BM progenitors (data not shown). We conclude that Tcf-1 is the sole Tcf/Lef family member expressed in HSC, and our data argue against activation of other Tcf/Lef factors to compensate for the lack of Tcf-1. The question thus remains: is there functional significance to the Tcf7 transcripts detected in HSC?

Perhaps yes, if we consider that *Tcf7* could be expressed as part of a lymphoid priming signature. Although the reason why this occurs still remains unclear, it is known that lymphoid specific genes are transcribed at low levels in the HSC (see *Introduction*). Examples of lymphoid specific gene transcripts expressed in HSC include *Notch1* and *Il7ra* (Ng et al., 2009; Yoshida et al., 2006). *Tcf7* could also be part of a lymphoid priming signature, but its presence might not be strictly required.

Yet another explanation is that the HSC defined here ($Lin^-c\text{-}kit^+Sca1^+Flt3^-$) is contaminated with an as yet un-described NK progenitor. We have also demonstrated in this report that *Tcf7* is expressed in all NK precursors examined, including the NKP and iNK. A recent report described an NK-committed progenitor negative for surface expression of IL-2Rβ, Flt3, and mature lineage markers (Fathman et al., 2011). However, unlike the HSC, the pre-NKP cell described is negative for surface expression of c-kit. The developmental stage preceding the pre-NKP is the $Ly6D^-$ CLP, which is negative for Flt3 but intermediate for expression of c-kit (**Figure 1.3**). It could be that the recently described pre-NKP or an intermediate between CLP and pre-NKP contaminated the HSC population as gated for qPCR analysis.

While we did not observe a defect in Tcf-1 deficient HSC and their progeny in the BM, it is possible that the defects conferred by Tcf-1 deficiency are not manifested in surface phenotype, cellular distribution, or proliferation. Rather, they could be less obvious and comprise criteria not measured here. For example, we did not measure self-renewal, a process that has previously been reported to depend on Wnt signals. To measure the self-renewal capacity of *Tcf-1*$^{-/-}$ HSCs would require elaborate experiments involving serial BM reconstitution and single-cell cultures to assess lineage potential. While we have already demonstrated here that total BM progenitors from Tcf-1 donors are defective in their ability to give rise to T and NK cells, further study by serial transplant could demonstrate a more profound defect in HSC self-renewal, as measured by the maintenance of pluripotency.

6.2 Tcf-1 is expressed in the ETP and is cell-intrinsically required for T-cell development starting at this stage.

We report here that Tcf-1 is not required for progenitor emigration from the bone marrow, as control and *Tcf-1*$^{-/-}$ mice had comparable frequencies of circulating progenitors with T-cell potential like the LSK, CLP and CTP. *Tcf7* expression is upregulated in the earliest detectable thymic progenitor (**Figure 3.1**), Tcf-1 deficiency produced a striking thymic defect, with a 10-fold decrease in thymic cellularity and a markedly altered distribution of progenitor subsets in

the thymus (**Figure 4.2**). Importantly, we show that the *Tcf-1*$^{-/-}$ thymic defect occured in the ETP, and thus at an earlier developmental stage than previously reported (**Figures 4.2** and **4.3**). Moreover, this defect was cell-intrinsic, as demonstrated by mixed BM chimeras (**Figure 4.12**). It is currently unclear why, despite the presence of ETPs in competitive chimeras, only few ETPs were detected in thymi of constitutive *Tcf-1*$^{-/-}$ mice. This may indicate defects in the *Tcf-1*$^{-/-}$ stroma cells that compromise the maintenance of the TSPs in these mice. Alternatively, the presence of WT thymocytes in the competitive chimeras may provide an environment that improves the survival of *Tcf-1*$^{-/-}$ TSPs. The complete block at the ETP stage seen in BM chimeras also contrasts with the development of small numbers of T cells in *Tcf-1*$^{-/-}$ mice (Schilham et al., 1998; Verbeek et al., 1995). The presence of more mature developmental stages in *Tcf-1*$^{-/-}$ thymi may reflect compensatory expansion of these intrathymic populations and has been previously noted in other mouse models (Krueger et al., 2010; Zlotoff et al., 2010).

Another factor important for T-cell development, as mentioned above, is Gata3. Interestingly, Gata3-deficient mice also have defects in the ETP compartment and at the DN2 stage. Tcf-1 and Gata3 were both speculated in an early report to play critical roles at the earliest stages of thymic development. Both *Tcf7* and *Gata3* showed marked upregulation in the FT at the embryonal stage corresponding to thymic seeding, and FTOC treated with antisense oligonucleotides to either *Tcf7* or *Gata3* blocked the development of Lin^{-}c-kit^{+} FT cells. Although Gata3 may be controlled by Tcf-1 in peripheral T cells to influence

Th2 (Yu et al., 2009), there still is no evidence for this relationship in developing T cells in the thymus. It is interesting to speculate upon the interaction between these two factors as their expression shows a strong positive correlation, and deficiency in either has shown multiple defects in T cells at various stages, including the ETP, DN2, the transition from DN3 to DN4, and in Th2 cells (Hosoya et al., 2009; Hosoya et al., 2010).

Interestingly, the ETP phenotype observed in *Tcf-1*$^{-/-}$ mice has also been reported in the genetic ablation of *Gfi1*. As in the case of Tcf-1 deficiency, loss of Gfi1 resulted in severely decreased thymic cellularity due to increased apoptosis though proliferation remained unaffected. *Gfi1*$^{-/-}$ mice also had increased Id2 expression, and Gfi1 was able to negatively regulate PU.1 (product of the *Sfpi1* gene) by direct binding to the URE of the *Sfpi1* gene (Spooner et al., 2009; Yucel et al., 2003). The PU.1 URE was also found to contain a putative Tcf/Lef site, and Tcf-1 was found to bind this motif by EMSA (Rosenbauer et al., 2006). Moreover, mutation of the Tcf-1 binding site increased the expression of a reporter gene in a transient transfection reporter assay, indicating an inhibitory function for Tcf-1 in the expression of PU.1. Deletion of the URE resulted in decreased DN2 and DN3 cells and an overall reduction in thymic cellularity (Rosenbauer et al., 2006). Tcf-1 and Gfi1 can both act as repressors, and both have been implicated in CD8 memory formation (Chandele et al., 2008; Jeannet et al., 2010; Zhou et al., 2010). While both are important for T-cell biology, a direct relationship has not been established between Tcf-1 and Gfi1, yet the

uncanny similarity of their defects throughout T-cell development may indicate coordinate regulation of the two to orchestrate similar processes.

Alternatively, the pleiotropic effects observed in mice deficient for Tcf-1, Gata3, and Gfi1, respectively, may be convergent manifestations of genetic deregulation in the ETP, independently caused by ablating these factors. The defects observed in later thymic stages or in the periphery could indicate either that these factors are necessary for all these developmental stages and are similarly regulated, or that the aberrations occurring in the early progenitor would propagate to all progeny cells. It is still unclear whether peripheral defects observed by others are a result of defects originating in the thymus. The use of mice carrying a conditional deletion in *Tcf-1* may help to clarify whether Tcf-1 is required in each of these distinct T-cell populations, or whether the defects observed are the effect of genetic deregulation at the ETP.

6.3 Tcf-1 is directly activated by Notch1.

Initiation of the T-cell program is marked by activation of Notch signaling by Delta-like ligands expressed on thymic stroma cells. The data presented here suggest that the next critical step in the signaling hierarchy of early T-cell specification is the induction of Tcf-1. We showed transcriptional upregulation of *Tcf7* upon *in vitro* exposure to Notch ligands as well as ectopic Notch1 activation. Moreover, we have identified a conserved element in the DNA sequence

upstream of the *Tcf-1* gene that is occupied by Notch, CSL and Tcf-1 itself. Tcf-1 has been previously reported to promote its own expression (Hovanes et al., 2001; Roose et al., 1999). These findings suggest that the identified region has enhancer properties and that *Tcf-1* is a direct target of Notch. Notch1 ablation has been shown to redirect thymocytes to the B-cell lineage (Wilson et al., 2001) and given the potential of Tcf-1 to function as a transcriptional repressor, it would be interesting to speculate upon roles of Tcf-1 to suppress alternate hematopoietic fates. Additionally, other reports suggest ectopic expression of Tcf-1 suppress B-cell and myeloid fates (Weber et al., 2011). However, since *Tcf-1* deficient thymi showed no accumulation of B cells, we conclude that Tcf-1 is not required for the suppression of the B-cell differentiation program. Tcf-1 may be required in progenitors that have already lost B-cell potential.

6.4 Tcf-1 is specifically required at the c-kit^{+} DN1 cell subset.

The microarray data reported here provide us with multiple potential Tcf-1 gene targets that could be validated and investigated further. Additionally, it would be interesting to see if the gene profiles of *Tcf-1*$^{-/-}$ thymic subsets downstream of the ETP indicate similar gene targets; in other words, does Tcf-1 ablation alter expression of the same set of genes at different developmental stages at which it is expressed?

Overall the expression profile of the c-kit^{lo} *Tcf-1*$^{-/-}$ ETPs is similar to that of control ETPs. However, a significant downregulation of pathways involved in DNA metabolism and response to DNA damage (as observed in our microarray analyses) may explain the increased fraction of apoptotic thymocytes observed in Tcf-1-deficient mice (**Figures 4.9A** and **4.9B**). Increased apoptosis could be the result of abnormal expression of pro- or anti-survival factors. However, mRNA expression of Bcl-family members, key mediators of cell survial and death in the thymus, was not significantly altered in *Tcf-1*$^{-/-}$ ETPs, although many survival factors are regulated at the protein level (**Figure 4.9**). Although it has previously been reported that *Il7ra* is induced by β-catenin/Tcf signals (Yu et al., 2007)here we show that loss of Tcf-1 upregulated *Il7ra* in ETPs. Our findings are consistent with the increased surface IL-7Rα expression previously noted in *Tcf-1*$^{-/-}$ DN1 cells (Goux et al., 2005). Their IL-7Rα^{+} c-kit^{lo} surface profile renders the *Tcf-1*$^{-/-}$ ETPs phenotypically similar to CLP, which have not previously been detected in the thymus. While this may suggest that a CLP-like cell population could enter the thymus at low frequency even in the wildtype but might be masked by more abundant subsets; further analyses will be required to address this possibility.

The upregulation of *Il7ra* we observed in our arrays of *Tcf-1*$^{-/-}$ ETPs (**Figure 4.9**) seems to indicate a possible role for the repression of IL-7R by Tcf-1. Aberrant upregulation of *Il7ra* could have profound effects on the development of T-cell progenitors. While signaling through IL-7 is important for proliferation in T cells, it is generally thought to inhibit thymocyte differentiation beyond the DN2 stage. Kawamoto and colleagues reported that LSK cells cultured in a feeder-cell

free system (feeder free, FF), under defined conditions of Notch-ligand and cytokines, were unable to progress beyond the DN2 stage until IL-7 concentration was reduced in the FF culture medium (Ikawa et al., 2010). During V(D)J recombination Rag proteins introduce DNA breaks to allow for the joining of non-adjacent gene segments by NHEJ. Unrepaired damage to the DNA could be detrimental to a dividing cell. If allowed to propagate, a cell with compromised genomic integrity could become cancerous. Downregulation of IL-7R is thought to prevent this by promoting cell cycle exit and TCRβ and TCRα-chain rearrangement (at the DN3 and DP stages). This invites a model in which Tcf-1 upregulation in response to Notch signaling contributes to inhibiting IL-7 signaling by downmodulation of IL-7R. This is unlikely to explain the total defect in Tcf-1 deficient mice because these animals exhibit an earlier developmental block than the DN2 stage and have a prominent DN3 population; however, a failure to repress IL-7R in *Tcf-1*$^{-/-}$ ETPs may contribute to the compound phenotype observed. An antagonistic relationship between IL-7 and Tcf-1 has previously been suggested. IL-7 signaling has been shown to result in the downregulation of Tcf-1, Lef-1, and RORγt, all of which are essential for the transition from ISP to DP (Yu et al., 2004). If Tcf-1 is required for downmodulation of IL-7R prior to rearrangement of TCR components, high IL-7R expression as a result of Tcf-1 deficiency could prevent differentiation to the DP.

In contrast to a recent report proposing that Tcf-1 may directly upregulate the expression of T-cell essential genes (Weber et al., 2011), our profiling of *ex vivo* kit^{lo} *Tcf-1*$^{-/-}$ and control ETPs argues that Tcf-1 does not generally affect the

expression of T-cell specification factors, at least at this early stage (**Figures 4.9B** and **4.9C**). Interestingly, gene profiling of human $CD34^+$ thymocytes, which correspond to the most immature DN1/DN2 populations in the mouse, in response to induced Wnt/Tcf signaling identified a core set of gene targets, of which many were involved in cell-proliferation, but none in T-cell differentiation. Furthermore, quantitative PCR revealed that several of the genes identified were downregulated in *Tcf-1*$^{-/-}$ mice (Staal et al., 2004). These data implicate Tcf-1 in cellular maintenance.

We did observe a few expression changes in lineage-specific genes, such as the upregulation of *Id2* and E2a (*Tcf3*) (**Figure 4.9C**). Id2 has been implicated in the differentiation of effector memory CD8 cells, while Tcf-1 has been implicated in central memory, providing some circumstantial evidence for an opposing relationship between Id2 and Tcf-1. Paradoxically, both *Id2* and *Tcf3* are upregulated in *Tcf-1*$^{-/-}$ progenitors, even though Id2 is a known repressor of E2A protein; however, this might indicate a regulatory feedback loop between Id2 and E2A. Further studies will be needed to determine the physiological significance of the few expression changes in T-cell specific genes we did observe.

6.5 Tcf-1 functions independently of the canonical Wnt signaling pathway.

Evidence presented here suggests that Tcf-1 may have a functional role in T-cell development that does not depend on its binding to β-catenin (**Figure 4.11**). This may indicate that Tcf-1 exerts its function at this early developmental stage mainly as an architectural DNA bending factor. Although it has been assumed that Groucho must be displaced by β-catenin during gene activation, the sites of β-catenin and Groucho binding on Tcf-1 do not overlap, and there is no strong evidence for mutual exclusivity in the binding of β-catenin and Groucho. Whether β-catenin and Groucho compete for binding to Tcf-1 might be addressed by an inducible expression system to show decreased binding of Groucho to Tcf-1 with increased β-catenin. In the meantime, our data could suggest that alternative mechanisms of Tcf-1 mediated gene regulation exist. *In vitro* studies have suggested that Tcf/Lef proteins are required to bring into proximity other transcriptional modulators, and it remains unclear how this functionality depends on β-catenin or Groucho. It could be enlightening to understand whether Tcf-1 isoforms lacking the β-catenin binding domain are expressed *in vivo* in the thymus and whether they might be fulfilling repressor functions, as has been suggested. Although our study of a reporter mouse strain suggests the presence of activation competent Tcf-1 in wildtype T-cell progenitors, there is currently no method for measuring repression activity of Tcf-1. Nevertheless, our data warrant future efforts along this novel avenue.

In conclusion, our findings establish Tcf-1 as an essential early responder to Notch signals that are critically required for further progression to the T-cell lineage. We show that in the absence of Tcf-1, progenitor thymocytes downregulate DNA metabolic processes and become abortive.

6.6 Tcf-1 is expressed in BM NK cell progenitors and is cell-intrinsically required for NK-cell development starting at the NKP stage.

Here we report a novel defect in NK cells due to loss of Tcf-1. Tcf-1 expression begins in the NKP and is maintained throughout all NK progenitors expressing IL-2Rβ (**Figure 5.3**). This is intriguing as the molecular networks involved in the specification and development of early NK progenitors are poorly defined. *Tcf-1*$^{-/-}$ mice have a 4-to-5 –fold decrease in the distribution and total cellularity of IL-2Rβ^{+} BM NK progenitors, including the NKP, iNK and mNK. We show that this defect is cell-intrinsic as mixed BM chimeras showed few, if any, BM NK progenitors and NK1.1^{+} cells in the spleen from Tcf-1 deficient donors (**Figure 5.4**).

The defects observed in both T and NK cell lineages, but not in B cells or macrophages, might suggest a progenitor downstream of the CLP with potential to give rise to the T and NK cell lineages that might be compromised in Tcf-1 deficient mice. Another possibility is the presence of distinct BM niches that allow for either NK or B cells to develop from CLP. In this scenario, environmental

cues in the NK BM microenvironment, like the thymic microenvironment, would instruct the upregulation of *Tcf7*. We report the direct regulation of *Tcf7* by Notch1 in the thymus. Whether Notch1 can enhance expression of *Tcf7* in developing BM NK cells, remains to be clarified. While Notch1 is expressed in CLP, the ligands available in the BM microenvironment may be limiting and thus distinguish 'B-' and 'NK-cell niches'. In the absence of definitive evidence for such a model, however, further study is required to establish whether Tcf-1 can and/or must be be induced by Notch1 in BM progenitors to allow for NK-cell development.

The observed defect in NK cell development also invites the speculation that Eomes, a modulator of IL-15 responsiveness, might be involved. IL-15 signaling is central to NK cell biology. It has been described that Eomes regulates mNK cell function by modulating IL-2Rβ expression and thus, IL-15 responsiveness (Townsend et al., 2004). Interestingly, a connection between Eomes and Tcf-1 has already been established in the development of memory CD8 cells, where Tcf-1 positively regulated Eomes (Zhou et al., 2010). Considering this angle, it will be important in the future to study whether such a relationship exists also in NK cells, and whether the control of IL-2Rβ expression by Tcf-1 is necessary for NK cell development.

Finally, the E-protein antagonist Id2, which was upregulated in our gene expression analysis of Tcf-1 deficient ETPs (**Figure 4.9C**), plays a critical role in the development of NK cells as a deletion in Id2 resulted in a severe reduction of mNK cells (Boos et al., 2007). Enforced expression of Id2 in FTOC cells blocked

T-cell development while stimulating NK cell (Fujimoto et al., 2007). ChIP-seq and global expression analyses are necessary to determine whether Tcf-1 regulates the same genes in NK progenitors and early thymic progenitors.

6.7 Perspectives and Future Directions

6.7.1 Does Tcf-1 regulate gene expression?

Given that Tcf-1 is broadly bound throughout the genome in DP thymocytes (unpublished data from our lab) it is interesting to understand whether this binding pattern is established at the earliest incident of Tcf-1 expression or whether its binding pattern gradually changes as the cell differentiates. Additionally, it would be revealing to understand in how gene expression changes correlate with Tcf-1 binding pattern. ChIP-seq experiments with DN cell populations, particularly the ETP, where *Tcf7* is first expressed in the thymus, in combination with microarray or RNA-seq studies, would allow us to correlate Tcf-1 promoter and URE occupancy with gene expression levels in the context of progression of T-cell progenitors to mature lymphocytes. Such experiments are, however, complicated by the fact that a variety of splice-isoforms and alternative transcripts have been described without there being any definitive data on which Tcf-1 splice variants are present at these developmental stages.

Presumably, the Tcf splice variant would determine the protein binding pattern as has been subtly suggested by an *in vitro* study showing that an alternative tail, rather than the core Tcf, dictated promoter specificity (Atcha et al.,

2007; Hecht and Stemmler, 2003). Moreover, there is evidence to suggest that different isoforms might recruit distinct subsets of modification factors to the site of binding (Giese et al., 1991; Roose et al., 1998; Van de Wetering et al., 1996). Taking this into account, it may be found that the genes repressed by Tcf-1 differ from the ones activated by Tcf-1. Indeed, several variants could be co-expressed, thereby fine-tuning the regulation and biological function of Tcf-1 at each distinct developmental stage.

6.7.2 Is Tcf-1 a "specification factor"?

Tcf-1 has been implicated in directing the expression of T-cell specific genes, but its preference for expression in T-cells and T-cell progenitors does not necessitate a role in specification. Multiple lines of evidence support the notion of lymphoid "priming," or the early expression of genes that are said to be "specific" to a given cell fate. Indeed the defining feature of pluripotent progenitors may be a basal activation of multilineage genes in a stochastic fashion (Strasser et al., 2012). As cells progress through development they become increasingly lineage "restricted". One might propose a model, in which lineage restriction is a result of preventing alternative fates. In this model of decreasing progenitor potential, it would be expected that critical "specification factors" be involved in repressing other cell fates. Indeed, both critical developmental checkpoints that 'cement' T cell fate are defined by the activity of Notch at the DN1 and Bcl11b at the DN2 stage, to repress the B- and NK-cell lineages, respectively. As for Tcf-1, on the other hand, our data suggest that it is dispensable for the repression of B-cell fate and is actually required for NK cell development. Thus we tend to reject the

notion that Tcf-1 specifies T-cell fate. Instead, we have presented evidence that while Tcf-1 is a gatekeeper of lymphocyte fate, it might function by ensuring cell survival rather than specifically instructing T-cell fate through regulation of a T-cell program.

A way to further support this interpretation and address whether Tcf-1 functions in the specification of T-lineage fate or rather in cellular maintenance and metabolic processes might become evident if we consider the evolutionary relationship between Tcf/Lef family members. Tcf/Lef factors have high levels of homology and are identical in their DNA-binding domains, and Tcf-1 and Lef-1 are thought to share a common ancestor in the chicken Tcf. Given this evolutionary conservation, it seems reasonable to hypothesize that all Tcf/Lef family members have similar basal functions but in various cell types. One could further rationalize that a widely similar pattern of DNA occupancy of different Tcf/Lef factors in different cell types would argue against a role in cell-fate specification in the sense described above. Significantly different binding patterns on the other hand could be consistent with a role in manifesting cell identity. Therefore, future studies might compare the DNA occupancy of other Tcf/Lef factors in the respective tissues in which they are expressed, as well as in T progenitors ectopically expressing other Tcf/Lef factors to complement Tcf-1 deficiency.

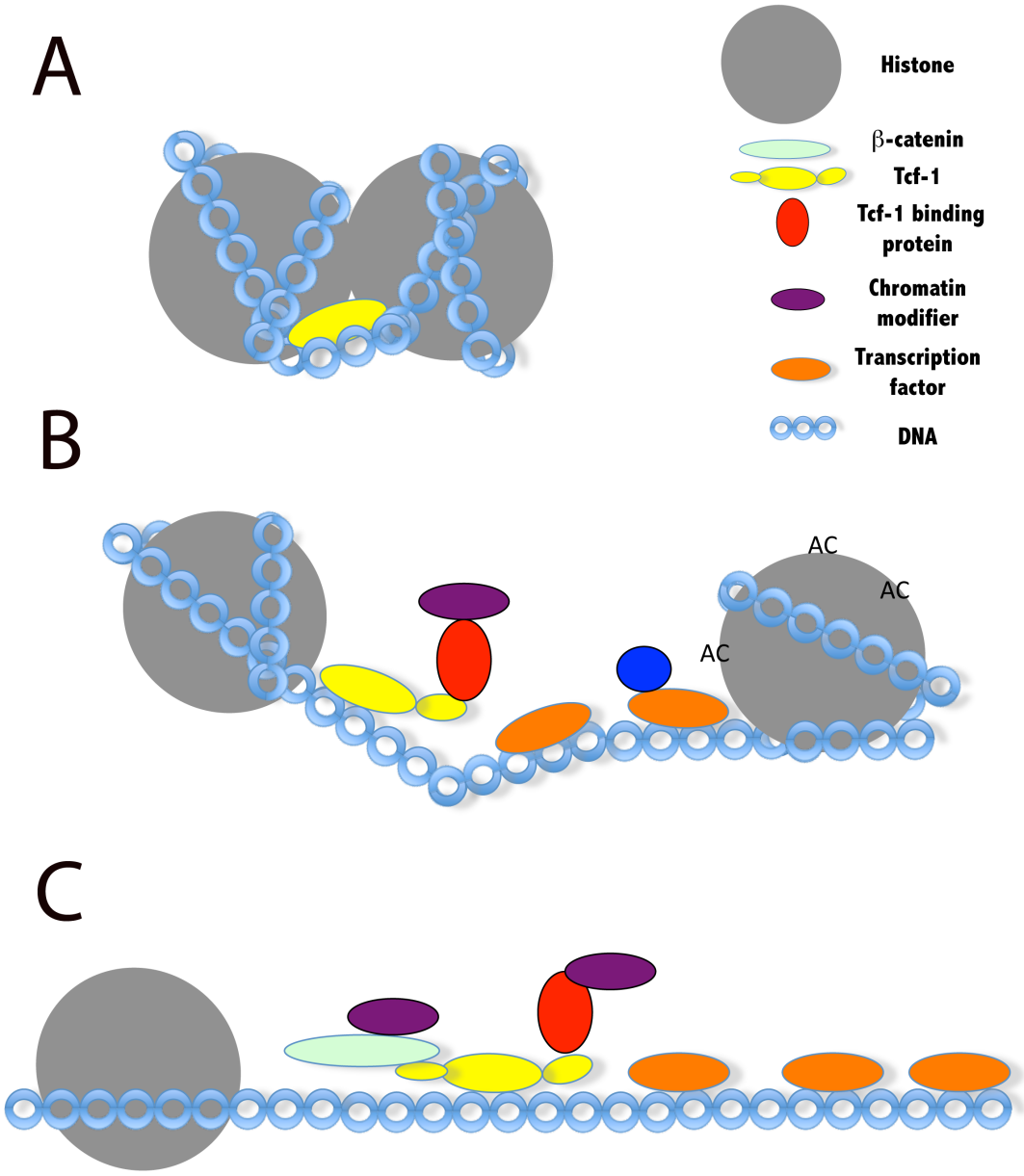

Figure 6:1: A model for Tcf-1 function. A. Short splice forms of Tcf-1 can act as strong DNA binding proteins but function as repressors because they do not contain the domains needed to interact with transcriptional activators. **B.** Tcf-1 interaction with an as yet unidentified factor could aid in the modification of the DNA architecture to allow the binding of other transcription factors. **C.** Binding of β-catenin to Tcf-1 would enhance the opening of DNA not necessarily in activating a specific set of genes.

I propose a role for Tcf-1 in maintaining the open chromatin conformations required for the dynamic transcriptional changes that occur during T-cell development. Rather than specifying lineage fate or controlling a specific set of genes, binding of Tcf-1 may be important for the recruitment and/or binding of other transcription factors near HMG boxes where Tcf-1 can be found. The shortest Tcf-1 isoforms lacking the capability of binding to a transactivator, could, even in the absence of Groucho, function as repressors given that they can bind DNA and potentially compete with “activating” Tcf-1 splice forms (**Figure 6.1C**). The potential for distinct functions and biological outcomes dictated by the predominant *Tcf7* isoform expressed warrants an in-depth characterization of the splice species that are present during development. It is likely that Tcf-1 interacts with proteins other than β-catenin, which would explain the rescue of Tcf-1 deficiency by transduction with a Tcf-1 transgene lacking the β-catenin binding domain. The identity of these protein partners or the domains on Tcf-1 with which they might interact remains to be discovered. Additional factors that might interact with Tcf-1 could also function to recruit chromatin modifiers and open the DNA (**Figure 6.1B**). Changes in chromatin conformation would thereby allow other critical transcription factors to bind enhancer sites and either activate transcription, or aid Tcf-1 in further opening the DNA. The potential for coordinated function with other factors described to be important for T-cell development may explain the overlapping phenotypes observed in loss- and gain-of-function models reviewed here. Binding of β-catenin would enhance the

capacity of Tcf-1 to open the DNA (**6.1C**). Modifying the architecture of regulatory sites within the genome is crucial to allow access for other factors to activate transcription. Thus, we predict that loss of Tcf-1 would result in a failure to open the chromatin gates, and thus inefficient transcription of various genes implicated in both T cell function and cellular maintenance.

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