Signaling events at the β-selection checkpoint during thymocyte development

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1 ZUSAMMENFASSUNG

Im Rahmen der T Zellentwicklung im Thymus sorgt eine Reihe von Selektionsprozessen für den Aufbau eines funktionsfähigen T Zellrepertoires. Grundvoraussetzung für die Reifung innerhalb der αβT Zellinie ist ein produktives Rearrangement des T Zellrezeptor-β (TCRβ) Genlokus und die Zusammenlagerung eines prä-T Zellrezeptorkomplexes (preTCR) auf der Zelloberfläche. Der preTCR signalisiert konstitutiv und zellautonom Proliferation, Überleben und Differenzierung von unreifen Thymozyten und stellt deshalb einen wichtigen Kontrollpunkt in der frühen T Zellentwicklung dar, der auch als β-Selektion bezeichnet wird. Die Signalwege, die der Entwicklung von unreifen Thymozyten über diesen Kontrollpunkt hinaus zu Grunde liegen, sind allerdings größtenteils unbekannt. Neben dem preTCR sind noch andere Signale notwendig, um die T-Zellentwicklung voranzutreiben, die vermutlich von Epithelzellen im Thymus bereitgestellt werden.

Der onkogene Transkriptionsfaktor c-Myc wird in seiner Expression sowohl von Wnt als auch von Notch Signalen beeinflusst. In dieser Arbeit wurde daher das Zusammenspiel zwischen preTCR und c-Myc detailliert erforscht. Mäuse, denen aufgrund *Lck*Cre vermittelter Rekombination das *Myc* Gen in $CD44^{-}CD25^{+}$ DN3 Zellen fehlt, zeigten vermindertes Zellwachstum und verminderte Zellteilung, was sich in Hypozellularität mit einer 10- bis 50-fache Reduktion von $CD4^{+}CD8^{+}$ DP Zellen niederschlug. Im Gegensatz dazu wurden Zelldifferenzierung und –überleben durch die Abwesenheit von c-Myc nicht beeinträchtigt. Sowohl *in vivo* als auch *in vitro* entwickelten *Myc*$^{-/-}$ DN3 Zellen sich zu DP Zellen und exprimierten TCRαβ auf der Zelloberfläche ohne sich zu teilen. Diese Beobachtung wies auf eine Verzweigung verschiedener Signalwege am preTCR Kontrollpunkt hin.

Die Serin-/ Threoninkinase Akt ist für verschiedene Aspekte des Überlebens von Zellen in vielen Organsystemen wichtig. In dieser Studie wurde die Rolle von Akt Signalen in der β-Selektion untersucht. Es wurde beobachtet, dass preTCR Signale Akt zwar aktivierten, dass aber die Expression einer konstitutiv aktiven Form von Akt (MyrAkt) nicht ausreichte, um die zusätzliche Abhängigkeit sich entwickelnder, adulter, muriner DN3 Zellen von Notch Signalen zu überwinden.

Diese Tatsache ließ darauf schließen, dass mehrere Signalwege das Überleben von Zellen an diesem Kontrollpunkt regulieren.

Ein weiterer Signalweg, der für die T Zellentwicklung eine Rolle zu spielen scheint, ist die Wnt/ β-Catenin Signalkaskade. Interessanterweise wurde hier festgestellt, dass die Stabilisierung von β-Catenin in DN3 Thymozyten die zügige Differenzierung zu DP Zellen zufolge hatte, und zwar auch in der Abwesenheit von Notch und preTCR Signalen, und damit den β-Selektionskontrollpunkt überwinden konnte. Die Abwesenheit von Notch Signalen beeinträchtigte allerdings die Zellteilung dieser Zellen teilweise. Diese Daten ließen den Schluss zu, dass Notch unabhängig von β-Catenin und dem preTCR Proliferationsignale sendet.

Insgesamt gesehen weisen die hier präsentierten Daten darauf hin, dass es unterschiedliche und unabhängige Signale gibt, die in der β-Selektion für Zellteilung, Differenzierung und Apoptose verantwortlich sind. Einige dieser Signalen könnten durch c-Myc integriert werden. Diese Arbeit läßt überdies auf ein komplexes System von Abhängigkeiten in der Regulation und Wechselwirkung der Signalwege schließen, die in die Entwicklung von DN zu DP Zellen im Thymus münden.

Schlagwörter: T-Zell Entwicklung, preTCR Kontrollpunkt, Signalwege

2 Abstract

During T cell development multiple selection processes ensure the generation of a functional and non-harmful T cell repertoire. Upon successful rearrangement of the T cell receptor (TCR) β gene immature thymocytes pass the first developmental checkpoint, also called β-selection. Productive TCRβ gene rearrangements lead to expression of the pre-T cell receptor (preTCR) and signals emanating from the preTCR promote proliferation, survival, and differentiation of immature thymocytes. However, the molecular mechanisms mediating the developmental progression past this β-selection checkpoint remain largely elusive. While preTCR signaling is required for proper thymocyte development it is not sufficient, and thymocytes need additional signals likely provided by thymic epithelial cells. Wnt/ β-catenin, Notch, and Akt signaling have been implicated in several stages of thymocyte development.

c-Myc is a target of Wnt as well as Notch signaling. We examined in detail the role of c-Myc specifically at the preTCR checkpoint. Employing *Lck*Cre mediated thymocyte specific ablation of c-Myc in $CD44^{-}CD25^{+}$ DN3 murine thymocytes we observed reduced proliferation and cell growth at the preTCR checkpoint, resulting in thymic hypocellularity and a severe 10-50 fold reduction in the number of $CD4^{+}CD8^{+}$ DP thymocytes. In contrast, c-Myc deficiency did not inhibit preTCR mediated differentiation or survival. *Myc*$^{-/-}$ DN3 cells progressed to the DP stage and upregulated TCRαβ surface expression in the absence of cell proliferation *in vivo* as well as *in vitro*, revealing for the first time a bifurcation of signaling pathways at the preTCR checkpoint.

The serine/ threonine kinase Akt has been implicated in cell survival in multiple aspects of T cell biology as well as other organ systems. Here, we addressed the role of Akt signaling at the β-selection checkpoint. We found that while Akt was activated in response to preTCR signaling, expression of a constitutively activated form of Akt (MyrAkt) was not sufficient to overcome the requirement for Notch signals in adult, murine DN3 cells, suggesting that multiple pathways mediate survival at this checkpoint.

Another major signaling pathway that has been implicated in the development of T cells is the Wnt/ β-catenin cascade. Interestingly, we found that stabilization of

β-catenin promotes rapid differentiation of DN3 thymocytes to the DP stage, even in the absence of Notch and preTCR, thus effectively overriding the β-selection checkpoint. Proliferation of these cells, however, was partially impaired in the absence of Notch, indicating that Notch provides proliferation signals independent of β-catenin and preTCR during β-selection.

Taken together, our findings indicate that there exist distinct signals downstream of the preTCR that are responsible for proliferation, differentiation, and apoptosis, some of which may be integrated by c-Myc. This study points to a complex interdependency of multiple signaling pathways resulting in DN to DP transition of immature thymocytes.

Keywords: T-cell development, preTCR checkpoint, signaling events

3 INTRODUCTION

3.1 The thymus

Despite its important function in the development of a potent immune system the thymus remained a mystery to scientists and physicians until well into the 20th century. Like no other major organ the thymus has given rise to speculations as to what its function might be, many of them placing it somewhere between an endocrine organ of unknown significance and an evolutionary remnant with no function whatsoever (Miller, 2002). Jacques Miller, who had initially set out to study murine lymphoblastic leukemia, discovered to his surprise that the thymus has a crucial immunological function (Miller, 1961). He demonstrated that thymus-derived (T) lymphocytes are a distinct and essential component of the adaptive immune system (Miller, 1962). These data eventually led to the discovery of the two major subsets of lymphocytes, the thymus-derived T and the bone marrow-derived B cells, and their interaction, providing a starting point for myriads of ensuing research studies addressing the mechanisms underlying immunological phenomena like memory, tolerance, autoimmunity, and genetically determined disorders (Miller, 2002). Recent data lend support to older observations from neck sections of mice suggesting the existence of a second, cervical thymus in the mouse ((Dooley et al., 2006; Law et al., 1964; Terszowski et al., 2006), a finding that might lead to further clarification of remaining puzzling observations in thymectomized mice as well as contribute to the elucidation of lymphopoiesis and the mediation of self-tolerance in general.

Many of the complexities of thymic architecture as well as thymocyte development have been intensively investigated during the past decades. We now know that the developing fetal thymus is colonized by lymphocyte progenitors in two waves between embryonic day 11 (E11) and E13, and in a second wave between E18 and E21 (Douagi et al., 2002; Misslitz et al., 2006). Postnatal lymphopoiesis on the other hand requires a continued influx of bone marrow-derived thymus-seeding progenitors (TSPs). These precursor cells depend on thymic epithelial cells (TECs) to differentiate into mature T cells. Within the thymus two main compartments are distinguished, the cortex and the medulla, both of which harbor several distinct TEC subsets, creating discrete

microenvironments that support different stages of development. During their development, immature thymocytes migrate within the thymus and interact with TECs, not only to their own developmental benefit but interestingly also to that of the stromal cells they encounter. In fact, a bi-directional interplay between developing thymocytes and stromal cells appears necessary to maintain intact thymic compartmentalization, and proper T cell development (Anderson and Jenkinson, 2001; Misslitz et al., 2006).

Immature thymocytes do not express the CD4 or CD8 coreceptors, and are therefore referred to as double-negative (DN) cells. After they complete their journey through the cortex DN cells will express a T cell receptor (TCR) on the cell surface, and have undergone rearrangement of the TCRβ as well as the TCRα genes. Before they are released into the peripheral circulation, however, they must pass positive and negative selection. These processes ensure the survival of only those T cells that recognize antigen presented by self-MHC but do not interact too strongly with self molecules, thereby reducing the risk of autoimmunity. While positive selection takes place in the cortex, the medulla seems to be crucial for negative selection, with only positively selected cells entering it.

While many of the mysteries the thymus evoked have been solved within the past 50 years, others have emerged. Establishing the thymus as the major site of T cell generation has certainly answered questions addressing its overall significance and function. Meanwhile, it has also given rise to more detailed inquiries about the molecular processes governing thymocyte development.

3.2 Getting there- extrathymic precursors in bone marrow and blood

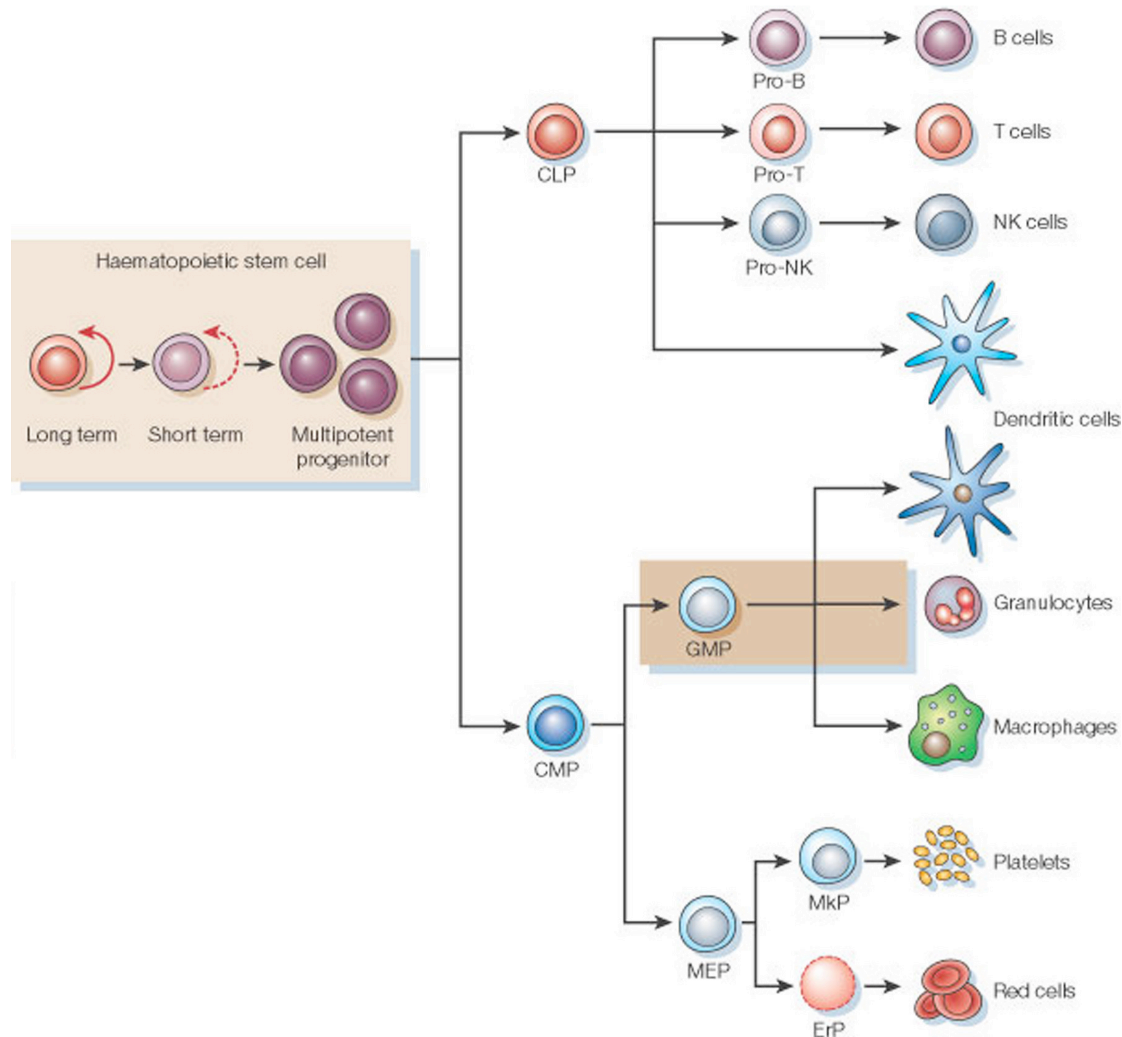


Figure 3.1: The classic model of hematopoiesis. HSC can be subdivided into long-term self-renewing HSC, short-term self-renewing HSC and multipotent progenitors (red arrows indicate self-renewal). They give rise to common lymphoid progenitors (CLP; the precursors of all lymphoid cells) and common myeloid progenitors (CMP; the precursors of all myeloid cells). Both CMP/GMP (granulocyte macrophage precursors) and CLP can give rise to all known mouse dendritic cells. Alternative models are discussed in the text. ErP, erythrocyte precursor; MEP, megakaryocyte erythrocyte precursor; MkP, megakaryocyte precursor; NK, natural killer. Reprinted from *Reya T, Nature 414, 105-111*

All adult blood cell lineages are derived from hematopoietic stem cells (HSC) that reside in the bone marrow and are part of the Lineage (Lin)$^-$ Sca1$^+$ c-kit$^+$ (LSK) population. These cells have the ability to self-renew indefinitely, and give rise to different lineages through a cascade of subsequent losses of developmental potential, a process also referred to as lineage commitment (Bhandoola and Sambandam, 2006). The capacity for self-renewal of HSC is lost upon upregulation of the cytokine receptor fms-related tyrosine kinase 3 (Flt3) while these multipotent progenitors (MPP) or LSK Flt3$^+$ cells retain their ability to generate granulocytes, monocytes, B and T cells (Adolfsson et al., 2001). In conflict with the current model of adult hematopoiesis (**Figure 3.1**) (Reya et al., 2001), which states that the first lineage commitment step of MPP leads to two strictly separated sets termed common myeloid and lymphoid progenitors (CMP (Akashi et al., 2000) and CLP (Kondo et al., 1997)) recent evidence suggests that a significant percentage of LSK Flt3+ cells has already lost its ability to generate erythrocytes and megakaryocytes before giving rise to a CMP phenotype (Adolfsson et al., 2005). These data have in turn been challenged, and have been attributed to inadequate timing, and a small number of cells used for transfer experiments giving rise to the contradictory observations (Forsberg et al., 2006). However, evidence from mice that express the green fluorescent protein (GFP) as a reporter of Ikaros activity, supports a model by which a major bifurcation 'downstream' of the short-term HSC gives rise to either an erythro-myeloid progenitor (CMP) or a lympho-myeloid progenitor (LMPP) as a gateway into two independent myeloid pathways (Yoshida et al., 2006). Ikaros is a member of the protein family of Krüppel-type zinc finger DNA-binding factors and is essential for normal lymphocyte development and homeostasis (Georgopoulos et al., 1994). CLP express high levels of IL-7 receptor alpha (IL-7Rα) but low levels of Sca1 and c-kit, and derive from MPP via a pathway that requires Flt3 ligand (Sitnicka et al., 2002) and Ikaros (Allman et al., 2003). Other lymphocyte progenitor populations found in the bone marrow are Rag-1$^+$ early lymphocyte progenitors (ELP) (Igarashi et al., 2002), L-Selectin$^+$

progenitors (LSP) (Perry et al., 2004), and CLP derived Lin^- $c\text{-}kit^{-/lo}$ $B220^+$ CLP-2 cells (Martin et al., 2003; Scimone et al., 2006).

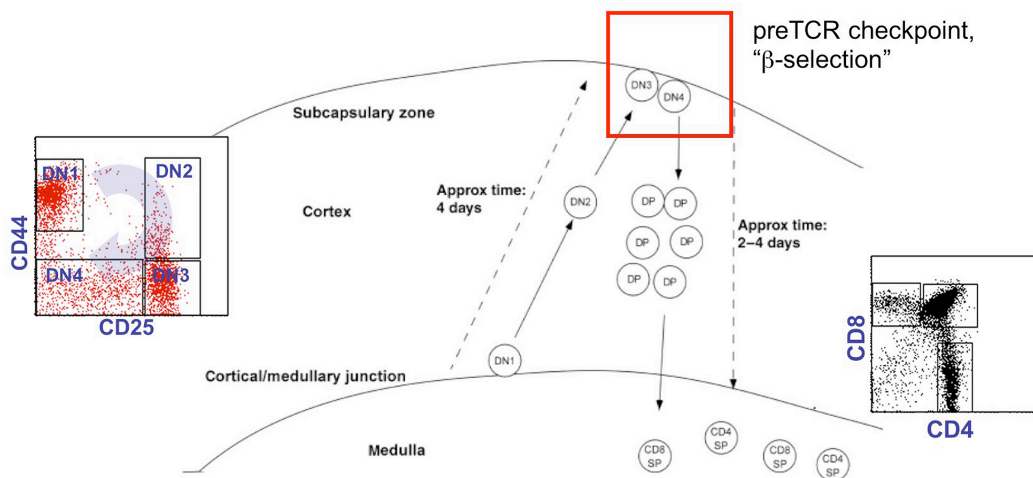
Classical transfer experiments have shown that sustained thymocyte development in the adult requires the continuous immigration of bone marrow derived precursor cells into the thymus via the bloodstream (Wallis et al., 1975). However, the nature of the circulating progenitors remains under investigation. A recent study employing intravenous injection of a range of bone marrow derived progenitor subpopulations into non-irradiated recipient mice has shown that HSC cannot home to the thymus but MMP and CLP efficiently do so. This ability coincided with the expression of CCR9 (Schwarz et al., 2007). Likewise, the interaction between P-selectin (expressed on thymic epithelium) and its ligand PSGL-1 (expressed on progenitors) has been implicated in the homing of progenitors to the thymus. The expression of P-selectin appears to be regulated by the availability of progenitor niches in the thymus, suggesting a sensory mechanism to direct progenitor access (Rossi et al., 2005). CLP-2 cells that were identified in the BM of transgenic reporter mice expressing human CD25 (hCD25) under the control of the pre-T cell receptor α (*Ptcra*) promoter and enhancer may likewise constitute a population of thymic immigrants (Gounari et al., 2002; Martin et al., 2003). Such cells have a Lin^- $c\text{-}kit^{-/lo}$ $B220^+$ phenotype and originate from Lin^- $c\text{-}kit^+$ $B220^-$ $IL\text{-}7R\alpha^+$ CLP cells. CLP-2 efficiently enter the thymus upon intravenous transfer and have limited self-renewal capacity (Martin et al., 2003; Scimone et al., 2006). When cultured in the presence of Notch ligands CLP-2 upregulate c-kit, downregulate B220, and quickly become DN2 cells (Krueger and von Boehmer, 2007). Of note, early thymic immigrants after BM transfer are mostly $c\text{-}kit^-$ (Mori et al., 2001) and enriched for $B220^+$ cells (Martin et al., 2003). However, CLP-2 cells have not yet been detected in blood. Very recently a circulating T cell progenitor (CTP) has been characterized, possibly closing a gap between lymphocyte-restricted bone marrow resident progenitors and thymus resident progenitors. These CTP have a $c\text{-}kit^{lo}Thy\text{-}1^+$ phenotype thus resembling a pro-thymocyte population previously described in fetal blood (Rodewald et al., 1994). In contrast to BM CLPs and CLP-2, these

cells possess efficient T, but only very limited B and NK potential and give rise to a single wave of T-cell development when transferred into irradiated mice (Krueger and von Boehmer, 2007).

In summary, it is clear that adult thymocyte development requires continuous seeding by bone marrow derived progenitors. Whether these progenitors are committed to the T cell lineage before they enter the thymus or not remains under investigation. While several bone marrow derived precursor populations can reconstitute the thymus and have been detected in adult blood, only CTP are committed progenitors and it remains to be addressed if there are others.

3.3 Inside the thymus- stages of T cell development

Within the thymus the most immature thymocyte subsets are mostly devoid of CD4 and CD8 expression and are therefore referred to as double-negative (DN) populations (Ceredig et al., 1985; Godfrey et al., 1994; Godfrey et al., 1993; Pearse et al., 1989). They can be subdivided based on the differential expression of the CD44 adhesion molecule and the IL-2 receptor alpha (IL-2Rα) chain CD25 (**Figure 3.2**). In this scheme the $CD44^{+}CD25^{-}$ DN1 population represents the most immature stage followed by the $CD44^{+}CD25^{+}$ DN2 population. The heterogeneous DN1 population comprises progenitors harboring B, T, myeloid, natural killer (NK), and dendritic cell (DC) potential, although reports vary as to which lineage potential is found, indicating that the lineage commitment process



Modified after: Robey and Bousso (2003), Immunological Reviews 195 (1), 51-57.

Figure 3.2: Progression of intrathymic development. DN1 precursors enter the thymus and progress outward through the cortex as they differentiate. FACS plots show the surface profile of the indicated subsets. After β-selection takes place in the subcapsulary zone thymocytes reverse their direction of migration.

might be highly sensitive to the microenvironment, cell numbers, and other variables (Martin et al., 2003; Matsuzaki et al., 1993; Porritt et al., 2004; Radtke et al., 1999; Wu et al., 1991). A closer look at the DN1 population revealed a subset termed early T lineage progenitors (ETP) characterized as Lin^- Sca^+ c-kit^{hi} IL-7$R\alpha^{lo/neg}$ that had T lineage potential upon intrathymic injection into recipient mice (Allman et al., 2003). ETP are also referred to as c-kit^+ $CD24^-$ DN1a and c-kit^+ $CD24^{lo}$ DN1b cells by others (Porritt et al., 2004). DN1 and DN2 cells depend on the c-kit ligand stem cell factor (SCF, (Colucci and Di Santo, 2000)) and IL-7 for proliferation and survival. IL-7R signaling results in cell cycle entry and survival as a result of Bcl-2 upregulation in immature thymocytes (von Freeden-Jeffry et al., 1997). More recently it has been shown that while IL-7 is crucial for survival of DN1 and DN2 cells it is dispensable after TCRβ rearrangement, and even inhibits differentiation of thymocytes that have undergone β-selection (Balciunaite et al., 2005). $\gamma\delta$ T cell development on the other hand seems to specifically require IL7-R signaling (Jiang et al., 2007; Malissen et al., 1997; Perumal et al., 1997).

$CD44^-CD25^+$ DN3 stage cells are arrested and undergo TCRβ rearrangement at the so called pre-T cell receptor (preTCR) or β-selection checkpoint until they express a functional preTCR, comprising a rearranged TCRβ as well as the generic pTα chain and CD3 subunits on the cell surface. Alternatively, if cells rearrange productive TCRγ and δ chains first, they develop into $\gamma\delta$ T cells (Livak et al., 1995). $\gamma\delta$ and $\alpha\beta$ T cells seem to diverge into these distinct lineages at the β-selection checkpoint (Rodewald and Fehling, 1998). It is at present not clear whether $\gamma\delta$ versus $\alpha\beta$ lineage fate is instructed by distinct receptor signals or whether the receptor signals confirm independently determined lineage fate. It is known, however that precursors of both lineages undergo $\gamma\delta$ as well as TCRβ rearrangement. Under physiological conditions $\alpha\beta$ lineage cells are selected against in frame $\gamma\delta$ rearrangements (Dudley et al., 1995; Livak et al., 1995), while $\gamma\delta$ T cells are selected against in frame TCRβ rearrangements (Aifantis et al.,

1998). These facts are consistent with either a TCR “instruction” or TCR “confirmation” model of lineage commitment.

Observations that favor a TCR-instructed lineage model are that pTα$^{-/-}$ mice display increased numbers of γδ T cells (Fehling et al., 1995) and that the frequency of TCRβ-expressing γδ T cells is doubled in pTα$^{-/-}$ versus wild-type mice. Single cell PCR experiments and analysis of intracellular TCRβ expression showed that little or no selection against in-frame TCRβ rearrangements occurred in γδ cells from pTα deficient mice, whereas pTα competent mice displayed reduced percentages of in-frame rearranged TCRβ alleles in γδ cells. These data suggest that signaling by the pre-TCR can prevent cells from entering the γδ lineage (Aifantis et al., 1998).

Observations that favor a TCR-independent commitment step are based on heterogeneous IL-7 receptor (IL-7R) levels on CD44+CD25+c-kit+ (DN2) pro-T cells that have not yet undergone TCR rearrangements (Kang et al., 2001) and suggest that lineage commitment can occur, at least in part, prior to TCR expression. Recently, heterogeneous expression of the transcription factor Sox13 has been implicated in TCR-independent lineage commitment (Melichar et al., 2007). Thus, the mechanisms governing lineage choice and commitment remain under investigation.

3.4 Migration of developing thymocytes

Developing thymocytes migrate from their point of entry, the postcapillary venules near the cortico-medullary junction, towards the subcapsular zone (SCZ) (Lind et al., 2001). Progenitor cells progressively undergo differentiation during their outward migration through the cortex, such that distinct developmental stages are localized in specific regions (see **Figure 3.2**). Cells that have passed the pre-TCR checkpoint, and have therefore successfully completed β-selection, then reverse their direction of migration and are guided back towards the medulla. Mature, positively selected single-positive (SP) cells enter the medulla and are released into the bloodstream (Petrie, 2003).

The migratory pattern during differentiation appears to depend on factors that ensure cell-matrix interactions (Prockop et al., 2002) as well as factors that provide directional information. Chemokine signaling has been shown to play a role in maintaining the orderly developmental progression of early thymocyte progenitors as well as thymic architecture. Such, progenitor cells deficient for the chemokine receptor 7 (CCR7) show disrupted localization and differentiation in CCR7 deficient as well as competent thymic stroma. A $CD25^{int}CD44^{+}$ (DN1-2) subset of CCR7 deficient DN thymocytes accumulates at the cortico-medullary junction (Misslitz et al., 2004). Likewise, adult $CCR9^{-/-}$ bone marrow does not repopulate the thymus of lethally irradiated mice as efficiently as wt bone marrow (Uehara et al., 2002), and CCR9 deficiency leads to abnormal intrathymic localization of DN2 and DN3 stage thymocytes throughout the cortex as opposed to the SCZ (Benz et al., 2004). In CCR7/ CCR9 double-deficient mice thymic seeding is impaired before vascularization during embryonal development but appears normal later in life (Liu et al., 2006). Ablation of the G-protein-coupled seven-transmembrane span receptor CXCR4, that specifically interacts with alpha-chemokine stromal derived factor-1 (SDF-1, CXCL12), in the thymus leads to a severe block at the DN1 stage and prevents homing of blood-borne precursor cells into the cortex, where they would normally undergo further differentiation (Plotkin et al., 2003). Chemokines are therefore important for guiding thymocytes during development.

3.5 The preTCR

Immune receptors have been known for almost as long as cells with immune function have been characterized in detail, i.e. for most of the past three decades. But even after mature B and T cell receptors had been described, their role in immature thymocyte development remained elusive. The TCRβ chain was subsequently characterized as an entity expressed on DN cells that was able to evoke characteristics of DN developmental progression, i.e. the downregulation of CD25 and progression to the DP stage. TCRβ was detected on DN thymocytes in the absence of any other (known) TCR molecule, giving rise to speculations as to its stoichiometric expression and hypothetical complex partners (Groettrup et al., 1992; von Boehmer and Fehling, 1997). In an important study Tonegawa and colleagues showed that mutations in the TCRα versus the TCRβ chain affected thymocyte development in different ways. More specifically, these authors found that TCRβ was needed much earlier in development than TCRα. This study also formally showed that γδ T cells represent a distinct lineage that does not depend on TCRα or β chains (Mombaerts et al., 1992).

Taken together these observations prompted the idea of an immature T cell receptor or preTCR comprising a productively rearranged TCRβ chain and supposedly other molecules.

3.5.1 The pTα chain

Indeed, the mystery of the seemingly “unpaired” expression of the TCRβ chain on DN cells unraveled when a novel component was identified, that formed a complex with TCRβ; this was the discovery of the generic pre-T cell receptor α (pTα) chain (Groettrup et al., 1993). Von Boehmer and colleagues proceeded to clone and characterize the gene encoding this novel surface molecule, establishing that it is indeed a type I transmembrane protein that forms disulphide-linked heterodimers with TCRβ chains. Sequence analysis revealed

that pTα consists of a single immunoglobulin-like extracellular domain, a transmembrane region, and a carboxy-terminal cytoplasmic region of ~30 amino acids (Saint-Ruf et al., 1994). Abolishing pTα expression in mice proved its essential role in development of αβ T cells (Fehling et al., 1995).

The existence of an intracellular domain in pTα, was in contrast to the structure of mature TCR chains, which contain no such regions but depend on their coreceptors for intracellular signal mediation. Initially, it was found that a tailless pTα construct was able to rescue the developmental defect of pTα$^{-/-}$ mice indicating that the cytoplasmic tail region was dispensable for preTCR signaling (Fehling et al., 1997). However, expression levels in this genetic complementation approach were about 50 fold above wt levels. The role of the cytoplasmic tail of pTα was therefore reexamined in detail in several systems including pTα$^{-/-}$ cells, mice, and cell lines that were modified to express different mutants of the pTα chain at more physiological levels. These refined analyses indicate that proline-rich motives in the pTα chain might be critical for its function (Aifantis et al., 2002), i.e. for proliferation, survival, and differentiation to the DP stage as well as the induction of a bi-phasic Ca^{2+} flux that results in the activation of the transcription factors NF-κB and NFAT in response to preTCR signaling (Aifantis et al., 2001).

It has also been demonstrated that pTα and TCRα are not interchangeable during thymocyte development, illustrating that the preTCR is a unique structure with specific signaling function that cannot be achieved by a mature αβTCR (Borowski et al., 2004). In addition to examination of functional moieties within the pTα cytoplasmic tail recent research also addressed differences in the extracellular domains of pTα and TCRα. preTCR constitutively colocalizes with $p56^{lck}$ Src kinase (Lck) in glycolipid enriched membrane domains (rafts). In contrast to mature αβTCR this happens apparently without any need for ligation (Saint-Ruf et al., 2000). These observations led to speculations about ligand independent receptor oligomerization being a key event in preTCR signaling. In fact, a recent study showed that pTα chains can spontaneously form oligomers,

and that a number of charged amino acids in the extracellular domain of pTα are necessary for this process. pTα chain mutants with alterations of these charged residues fail to rescue the β-selection defect observed in pT$\alpha^{-/-}$ mice (Yamasaki et al., 2006). These authors also show that overexpression of raft localizing CD3ε proteins is not sufficient to drive development whereas overexpression of CD3ε-dimers regardless of their localization is, arguing that pTα oligomerization results in CD3ε interaction and that the localization in rafts might be unrelated to the signaling capability of the receptor complex. Therefore the molecular basis of preTCR signal initiation remains under investigation, as does that of signaling events occurring further downstream.

3.5.2 preTCR signaling mediators

Several studies imply that the kinase p56^{lck} (Lck) mediates signals in early thymocyte development. Lck deficient mice show a similar developmental arrest as pT$\alpha^{-/-}$ and TCR$\beta^{-/-}$ mice, and transgenic expression of an activated form of Lck restores DP cell numbers to wt levels in pT$\alpha^{-/-}$ animals (Fehling et al., 1997). preTCR signaling results in the phosphorylation of the CD3ε subunit and the protein tyrosine kinase Zap-70 that is associated with CD3ε (Wange et al., 1993) and is activated by Lck phosphorylation in mature TCR signaling (Chan et al., 1995; Wange et al., 1995). Aside from CD3ε and Zap-70 phosphorylation NF-κB and NFAT mobilization have also been described as downstream events in response to preTCR signaling, but these findings are not sufficient to explain the multiplicity of events following β-selection.

Recent evidence both from loss and gain of function approaches indicates that several other genes and signaling pathways are involved in the preTCR checkpoint. These include kinases such c-Fyn (Groves et al., 1996), Csk (Kanazawa et al., 1996), and Pim1 (Jacobs et al., 1999), and adaptor proteins such as LAT and SLP-76(Pivniouk and Geha, 2000). Several transcription factors were also shown to be essential at this developmental stage such as Ikaros (Winandy et al., 1999), E2A (Engel and Murre, 2004), Runx2 (Vaillant et al.,

2002), and c-Myb (Bender et al., 2004; Pearson and Weston, 2000). Multiple findings indicate the involvement of a number of signaling cascades (Weerkamp et al., 2006a) including Notch (Deftos and Bevan, 2000; Wolfer et al., 2001), Wnt (van de Wetering et al., 2002a), and Hedgehog (Outram et al., 2000). The interactions between these pathways, however, the signals that mediate their activation and their orchestration with respect to preTCR signaling are largely unknown.

3.6 Notch signaling in thymocyte development

The Notch signaling cascade is an evolutionary conserved mechanism that controls cell fate decisions in many developmental processes (Artavanis-Tsakonas et al., 1999). Four Notch transmembrane receptors (1– 4) have been described that interact with ligands of the Jagged (1 and 2) or Delta-like (DL1, 3, and 4) family on neighboring cells. In the adult mouse thymus Notch signaling is initiated when Notch interacts with intrathymic DL ligands expressed on thymic epithelial cells. Recent evidence suggests that the predominant interaction is between Notch1 and DL4 (Besseyrias et al., 2007). Engagement of Notch receptor and ligand results in proteolytic cleavage by γ-secretase near the plasma membrane, allowing the intracellular domain of Notch (IC-Notch) to translocate into the nucleus (**Figure 3.3**). IC-Notch then binds to CSL (RBP-Jκ in mice), a transcription factor that mediates most of the well-characterized Notch functions, and converts it from a transcriptional repressor into an activator by displacing corepressors (Lai, 2002). In addition, IC-Notch binding to CSL results in the recruitment of a large multiprotein transcriptional activation complex. This latter function appears to be largely mediated by Mastermind-like (MAML) proteins (Wu et al., 2000), which likely function as scaffold components of the activation complex (Jeffries et al., 2002). Among the most consistently confirmed downstream targets of Notch are Hairy-Enhancer of Split (Hes) transcription factors (Gounari et al., 2002; Radtke et al., 1999) and Notch1 itself (Weerkamp et al., 2006b). Recently, a conserved CSL consensus sequence was found in the

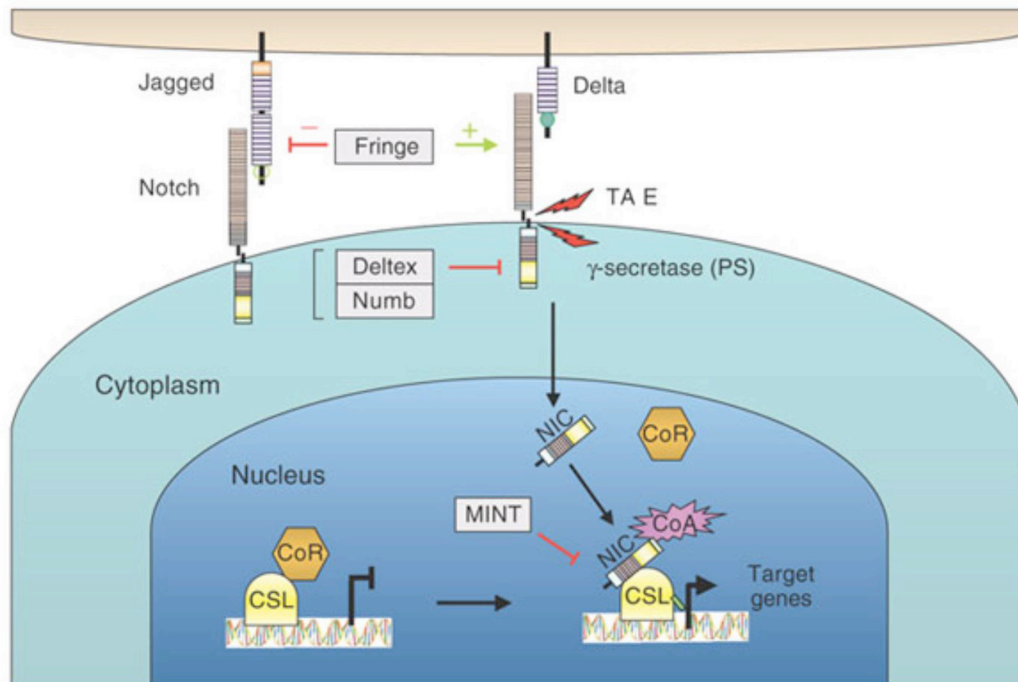


Figure 3.3: The Notch signaling cascade. The extracellular domains of transmembrane Notch receptors bind to ligands on the surface of neighboring cells. This sets of proteolytic cleavage of IC-Notch resulting in transcription of CSL responsive target genes. CoR, corepressor. Reprinted from *Radtke F, Nat Immunol. 2004 Mar;5(3):247-53.*

Myc gene encoding the transcription factor c-Myc, and it was shown that DN3 cells depend on Notch signals to maintain c-Myc expression (Weng et al., 2006). It has been shown that Notch1 receptor signaling is required for CD25 upregulation at the transition to the DN2 stage, confirming previous reports that commitment to the T cell lineage is dependent on Notch1 signaling (Radtke et al., 1999). Upon Notch1 deletion in hematopoietic cells these authors describe a dramatically enlarged DN1 compartment mostly comprising B cells that are found only in marginal numbers in wt animals, at the expense of mature thymocytes. Upon transfer of Notch1$^{-/-}$ bone marrow into irradiated wt mice only B cells

developed (Wilson et al., 2001). In a more recent study these results were essentially confirmed, and the need for Notch signals was assigned prior to the ETP stage, but after thymic seeding. This study employed a dominant negative form of MAML1 fused to the green fluorescent protein (GFP) (DNMAML1) to inhibit Notch signaling. DNMAML1 contains only the minimal binding sequence necessary for the interaction with IC-Notch, but lacks the domains required to recruit other components of the transcriptional activation complex. DNMAML1 was characterized as a potent inhibitor of all four Notch receptors known in mammals (Maillard et al., 2004). DNMAML1 expressing ETP could not compete with wt ETP while DNMAML1 expressing MPP were found at the expected levels in the blood, indicating that Notch signaling was required prior to the ETP but after the MPP stage (Sambandam et al., 2005).

Notch signaling is required at the DN3 stage at least until preTCR is expressed on the surface. Tissue specific Notch deletion after β-selection under the control of a *CD4*Cre transgene does not impact T cell development (Wolfer et al., 2001), but using an *Lck*Cre transgene for the conditional ablation of Notch1 at the DN2 to DN3 transition the same authors were able to detect a profound defect in TCRβ rearrangement and thymocyte development (Wolfer et al., 2002). In fact, the absolute requirement for Notch signaling at the β-selection checkpoint *in vivo* has recently been established by transfer of single cells from DNMAML1 transgenic mice into wt recipients, and residual development in previous mouse models appears to be largely due to delayed Cre mediated deletion. The latter finding is based on an elegant mouse model that results in simultaneous GFP and DNMAML1 expression upon Cre mediated deletion (Maillard et al., 2006). These authors also report that transgenic TCRs cannot compensate for the Notch deficiency, indicating that Notch as well as preTCR signals are needed in parallel at the DN3 stage. This notion has previously been conveyed by the discovery and characterization of an *ex vivo* coculture system that allows for *in vitro* differentiation of T cells from bone marrow as well as other immature precursor stages into mature T cells, employing OP9 stromal cells that have been transduced to express the Notch1 ligand Delta-like 1 (DL1) (Schmitt et al.,

2004; Schmitt and Zuniga-Pflucker, 2002). OP9-DL1 cells will only support differentiation of fetal liver derived Rag$^{-/-}$ DN3 cells if a TCRβ transgene is introduced (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005).
Notch signaling remains under intensive investigation with respect to the molecular basis for its effects on development at the DN3 stage. Some observations indicate that Notch1 signaling enhances proliferation of DN3 and DN4 cells on OP9 cocultures (Campese et al., 2006), and in fetal liver derived cells cultured on OP9-DL1 cells the growth-factor-regulated serine/ threonine kinase Akt (protein kinase B, PKB) has been described as a necessary requirement for Notch mediated survival (Ciofani and Zuniga-Pflucker, 2005).

3.6.1 Notch signaling in γδ T cells

Interestingly, all quoted reports describing impaired Notch signaling at the DN3 stage find that γδ T cells develop at normal levels in the absence of Notch. This has recently been substantiated by two *ex vivo* studies on OP9 cocultures, observing that while Notch signaling is needed for αβ lineage commitment, proliferation and differentiation, it only mildly if at all impacts γδ T cell development (Ciofani et al., 2006; Garbe et al., 2006; Taghon et al., 2006; Tanigaki et al., 2004). The one study proposes the existence of synergy between preTCR and Notch signaling which would provide a developmental advantage to preTCR competent DN3 cells, thus making preTCR signaling a more efficient way of generating αβ TCR DP cells (as opposed to a supposedly evolutionarily more ancient pathway of delineating γδ TCR^{+} cells back to the αβ lineage, a mechanism that seems to apply to about 10% of DP cells). These conclusions are based on the impact of Notch signal inhibition on T cell development *ex vivo* (Garbe et al., 2006). It was shown that the inhibition of Notch1 signaling activity does not inhibit γδ T cell development, and that preTCR^{+} cells out-compete TCR^{+} (preTCR^{-}) thymocytes in a competition experiment on OP9-DL1 cocultures. This competitive effect was significantly decreased as Notch signaling was inhibited. Another study finds that Notch signaling via the ligand Jagged1 favors the

development of NK and γδ T cells in the thymus (Lehar et al., 2005). Most cells, however, undergo β-selection, and mature further to the DP stage followed by rearrangement of the TCRα chain.

3.7 Wnt signaling in thymocyte development

Wingless-related mouse mammary tumor virus integration site 1 (Wnt) is the mammalian orthologue of the drosophila protein Wingless that has long been studied with respect to its role in segment polarity during embryogenesis (Nusslein-Volhard and Wieschaus, 1980) but is also important in many other species for development, differentiation, and tissue homeostasis (Logan and Nusse, 2004). Wnt signals can be transmitted via the transcriptional regulator β-catenin in a pathway referred to as canonical Wnt signaling. Canonical Wnt signaling is initiated by Wnt proteins, a family of secreted cysteine rich glycoproteins that bind to cell surface Frizzled receptors (**Figure 3.4**). In un-stimulated cells**,** newly synthesized β-catenin is captured in a large cytoplasmic complex consisting of the tumor suppressor APC (adenomatous polyposis coli), the constitutively active kinase GSK-3β (Glycogen synthase kinase 3β), and Axin. In this complex, β-catenin is phosphorylated by GSK-3β at four N-terminal serine and threonine residues and targeted for degradation (Aberle et al., 1997; Behrens et al., 1998; Ikeda et al., 1998; Kishida et al., 1998). Activation of the Wnt/ β-catenin cascade results in inhibition of the constitutive activity of GSK-3β (Cook et al., 1996) by the cytoplasmic protein Dishevelled (Dsv) (Itoh et al., 2000; Kishida et al., 1999; Noordermeer et al., 1994; Smalley et al., 1999). Consequently, β-catenin is no longer marked for degradation, accumulates in the cytoplasm, and translocates into the nucleus. Once in the nucleus, β-catenin binds to members of the T cell factor (Tcf)/ Lymphocyte enhancer factor (Lef) family of transcription factors (Staal and Clevers, 2003; van de Wetering et al., 2002a). Tcf/ Lef DNA binding factors are associated with Groucho proteins, forming transcription repressor complexes. β-catenin directly replaces Groucho

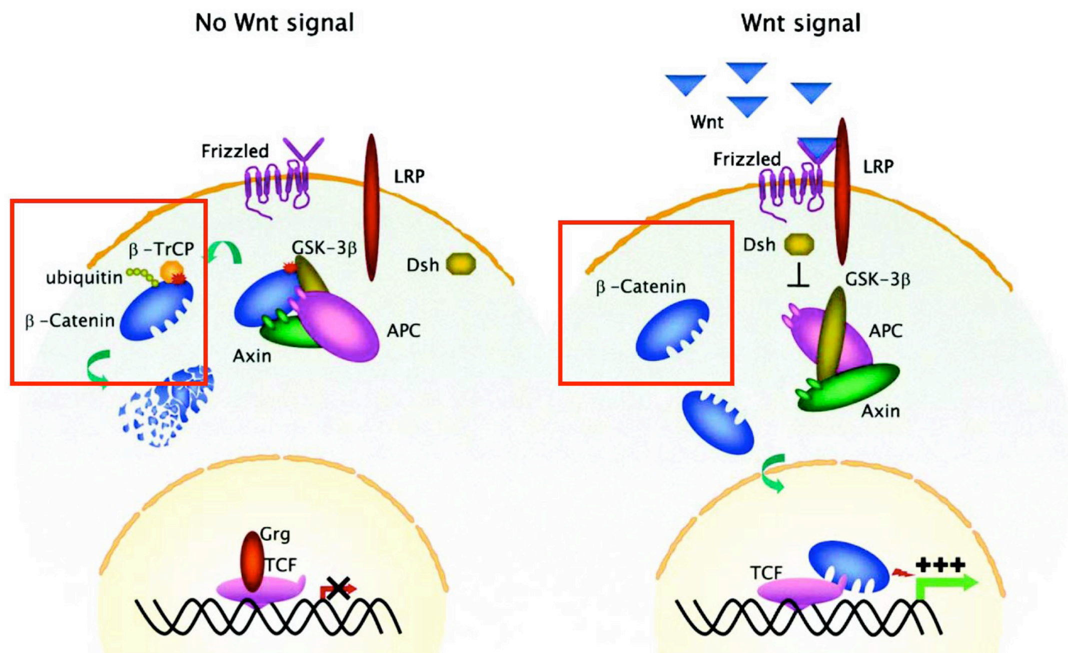


Figure 3.4: The Wnt/ β-catenin signaling cascade. Soluble Wnt molecules bind to Frizzled tranmembrane receptors resulting in Dsh mediated inactivation of the GSK-3β phosphorylation complex. In the absence of Gsk-3β phosphorylation β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it replaces groucho corepressors from transcription factors of the Tcf/ Lef family, resulting in gene expression. *Reprinted from van de Wetering, 2002, Cell*

proteins from Tcf/ Lef proteins thereby switching the status of Tcf/ Lef to act as a transcriptional activator (Daniels and Weis, 2005).

The Wnt/ β-catenin signaling cascade has been implicated in multiple stages of hematopoietic development. It was proposed that Wnt signaling controls the self-renewal of HSC (Reya et al., 2003). More recently, it was shown that deregulated activation of this pathway enforced cell cycle entry in HSC leading to the exhaustion of the long-term stem cell pool and a multi-lineage developmental block (Kirstetter et al., 2006; Scheller et al., 2006). In the thymus, loss and gain of function studies have indicated that Wnt/ β-catenin signaling is required for at least two stages during thymopoiesis. Thymocytes express the Tcf1 and Lef1

effectors of the canonical Wnt/ β-catenin signaling pathway. Ablation of Tcf1 activity affects the proliferating DN2, DN4 and $CD8^{+}TCR\beta^{-}$ immature single positive (ISP) stages (Schilham et al., 1998). The enforced expression of inhibitors of Wnt signaling, such as soluble Frizzled or Dickkopf proteins, in T-cell progenitors leads to a specific block in the DN1 to DN2 developmental transition (Staal et al., 2001; Weerkamp et al., 2006a). The concomitant ablation of Lef1 in a Tcf1 hypomorph mouse model results in a complete block of embryonic thymocyte development at the ISP stage (Okamura et al., 1998). The developmental block at the DN4 stage in Tcf1 deficient mice is β-catenin dependent since it can only be relieved by transgenic reconstitution with versions of *Tcf1* that contain an intact β-catenin binding domain (Ioannidis et al., 2001). Conditional ablation of β-catenin (Xu et al., 2003) or downregualtion of its activity by the expression of the inhibitor ICAT (Pongracz et al., 2006) have been shown to result in impaired DN to DP transition. Moreover, conditional stabilization of β-catenin at the DN3 stage of thymocyte development promotes development of DP and SP thymocytes. The developing DP cells in such mice have reduced TCRβ gene VDJ type rearrangements, and are largely devoid of preTCR and αβTCR (Gounari et al., 2001; Gounari et al., 2005). β-catenin stabilization in *Rag*$^{-/-}$ mice, i.e. in the complete absence of preTCR and TCR rearrangement, could still promote DN to DP transition, indicating that stabilization of β-catenin can circumvent the preTCR checkpoint. However, stabilization of β-catenin could not completely replace preTCR signals since the developing cells proliferated less and displayed increased cell death as compared to wild-type cells (Gounari et al., 2001).

3.8 c-Myc in thymocyte development

Overexpression of the basic region/ helix-loop-helix/ leucine zipper (bHLHZip) transcription factor myelocytomatosis oncogene (c-Myc) is probably the most uniform feature in hematopoietic malignancies and in particular Burkitt's lymphomas (Hoffman et al., 2002), as well as T-cell acute lymphoblastic leukemia (T-ALL) of the *HOX11* subtype (Ferrando et al., 2002). Transgenic mice overexpressing c-Myc develop both B and T cell lymphomas (Felsher and Bishop, 1999; Marinkovic et al., 2004; Spanopoulou et al., 1989; van Lohuizen et al., 1991). c-Myc has been established as a downstream target of the Wnt signaling pathway in the intestine (He et al., 1998; van de Wetering et al., 2002b). Very recently, c-Myc was shown to mediate most of the target gene inductions following the loss of the adenomatous polyposis coli tumor suppressor protein (Apc) that results in constitutive activation of β-catenin (Sansom et al., 2007). c-Myb, another Wnt/β-catenin target was shown to cooperate with c-Myc to cause T cell lymphoma in transgenic mice (Badiani et al., 1996). FRAT1 (frequently rearranged in advanced T-cell lymphomas) activity, which leads to the stabilization of β-catenin, synergizes with c-Myc to promote T-cell leukemogenesis (Jonkers et al., 1997).

c-Myc has also been implicated in lymphocyte development. Members of the Myc-family (c-Myc, N-Myc and L-Myc) play an integral role in proliferation, survival, and differentiation of normal and neoplastic cells. Myc binds E-box DNA motifs as a heterodimer with Max, resulting in cell cycle entry (Walker et al., 2005) and transcriptional activation or suppression of genes (Grandori et al., 2005; Levens, 2003; Nilsson and Cleveland, 2003; Pelengaris and Khan, 2003). c-Myc has been implicated in cell proliferation (Obaya et al., 1999) as well as the control of cell growth (Buckley et al., 2001; Iritani et al., 2002; Iritani and Eisenman, 1999; Johnston et al., 1999; Mateyak et al., 1997; Schuhmacher et al., 1999). Its expression increases rapidly in response to growth factors (Miyazaki et al., 1995; Waters et al., 1991), B-cell receptor (BCR) (Klemsz et al., 1989) or TCR (Lindsten et al., 1988) ligation. Immature B and T lymphocytes

express both c-Myc and N-Myc, while mature cells express only c-Myc.

A multitude of c-Myc target genes have been described, mostly by genetic screening approaches in systems in which c-Myc was artificially overexpressed. The results of these screens are available in a database for public use that is frequently updated and currently contains 1697 target genes (see http://www.myc-cancer-gene.org). Considering the large number of targets it becomes clear that some c-Myc targets are more relevant in certain processes than in others. c-Myc appears to regulate apoptosis in multiple ways and interestingly with multiple outcomes (Nilsson and Cleveland, 2003). Among c-Myc controlled genes regulating apoptosis are p53 tumor suppressor, Bcl-2, and Bcl-x_L (Eischen et al., 2001a; Eischen et al., 2001b; Maclean et al., 2003).

Several genes involved in cell cycle progression and growth control were shown to be transcriptionally regulated by c-Myc (Dang, 1999; Zeller et al., 2003) but the mechanism remains largely unclear. Some key cell cycle inhibitors such as $p27^{Kip}$, $p21^{Cip1}$, and Gadd45α have been described as negatively controlled by c-Myc (Cairo et al., 2005), and several D type cyclins appear to be transcriptional targets of c-Myc (Ausserlechner et al., 2004). c-Myc targets are thus under intensive investigation, but hard to determine. Given the large number of target genes obtained from c-Myc overexpression studies, it is of interest to confirm c-Myc targets in systems that are c-Myc deficient in the future.

Assessing the requirement for c-Myc in T-cell development was hampered in the past by the embryonic lethality of c-Myc deficient mice prior to the development of lymphocytes (Davis et al., 1993). To bypass this problem, Douglas and colleagues generated chimeric animals from *Myc*$^{-/-}$ ES cells and *Rag1*$^{-/-}$ blastocysts in which the *Rag1*$^{-/-}$ cells cannot contribute to the lymphoid lineages (Douglas et al., 2001). In this study *Myc*$^{-/-}$ progenitors populated embryonic thymi but had reduced proliferation and failed to develop beyond the late DN stages suggesting that c-Myc is essential for development past the preTCR checkpoint. c-Myc deficient cells did not populate adult thymi at all indicating additional defects at earlier stages of hematopoietic development. More recently, c-Myc has indeed been reported to control the self-renewal of hematopoietic stem cells

(Satoh et al., 2004), and its conditional ablation in the bone marrow favored self-renewal over differentiation of HSCs in the stem cell niche (Wilson et al., 2004). The involvement of c-Myc in early hematopoietic development indicates that studying its role at the preTCR checkpoint requires conditional animal models that avoid the accumulation of developmental defects resulting from c-Myc deficiency at earlier stages.

3.9 Aims

preTCR signaling is crucial for proliferation, survival, and differentiation of immature thymocytes that have undergone productive TCRβ gene rearrangements. While preTCR signaling is required for proper thymocyte development it is not sufficient, and thymocytes need additional signals likely provided by thymic epithelial cells to progress from the DN to the DP stage of development. The transcription factor c-Myc as well as its upstream effectors the Wnt/ β-catenin and Notch signaling cascades, and survival signals from the serine/ threonine kinase Akt have been implicated in several stages of thymocyte development. However, their exact role in and specific contribution to proliferation, differentiation and survival of thymocytes, like their potential interplay with each other and their regulation at the β-selection checkpoint are poorly understood. Our long-term goal is therefore to gain further insights into the signaling events at the β-selection checkpoint. The aims of this study were:

1) To dissect the requirements for c-Myc specifically downstream of the preTCR by answering first the question whether c-Myc is activated in response to preTCR signals, and second which branches of preTCR signaling outcomes are affected by *Lck*Cre mediated, stage specific ablation of c-Myc at the DN2 to DN3 thymocyte transition in mice. Furthermore it should be examined how, mechanistically, c-Myc might mediate its effects in β-selection.

2) To determine whether Akt is activated downstream of the preTCR and whether its constitutive activation in murine thymocytes may enable adult DN3 stage thymocytes to overcome the need for Notch signals for development past the β-selection checkpoint.

3) To establish how stabilization of β-catenin at the DN2 to DN3 thymocyte transition in mice may affect development past the preTCR checkpoint,

and whether β-catenin stabilization can drive development past β-selection in the absence of Notch as well as preTCR signals.

4) To analyze global gene expression changes in the absence or presence of c-Myc mediated proliferation upon preTCR signaling.

4 MATERIALS AND METHODS

4.1 *In vivo* experiments

4.1.1 Mice

All mice were kept under specific pathogen–free conditions in the animal facilities of Tufts-New England Medical Center according to protocols #49-03 and #22-06 approved by the Institutional Animal Care and Use Committee.

4.1.2.1 TetOβ

TetOβ transgenic mice were engineered by Fotini Gounari (Dose et al., 2006). A cDNA encoding the TCRβ chain of the 2B4 hybridoma was inserted into the polylinker of the TetO vector, a vector encoding 7 tet operator sequences linked to a minimal CMV promoter (Labrecque et al., 2001). A 2.9 kb *Xho*I fragment containing the tet operators, minimal CMV promoter, rat β-globin intron, TCRβ cDNA and rat β-globin polyadenylation signal was then used to generate transgenic TetOβ mice. These transgenic mice were crossed with LTH1 mice expressing a tetracycline transactivator (tTA) under the control of the proximal Lck promoter (Labrecque et al., 2001) to obtain TetOβ-LTH double transgenic mice. Subsequently, this strain was brought to the *Rag1*$^{-/-}$ background in order to abolish the expression of endogenous T cell receptors.

Figure 4.1 is a schematic representation of the interplay between the two transgenes in TetOβ-LTH *Rag1*$^{-/-}$ mice, and the effect of tetracycline. The TCRβ chain is expressed in the absence of tetracycline, resulting in the developmental progression of the otherwise arrested Rag1 deficient DN3 cells. In the presence of tetracycline the transactivator is not functional and the TCRβ chain therefore not expressed. Accordingly, thymic profiles of animals treated with tetracycline look like those of *Rag1*$^{-/-}$ mice, i.e. show a complete developmental block at the DN3 stage.

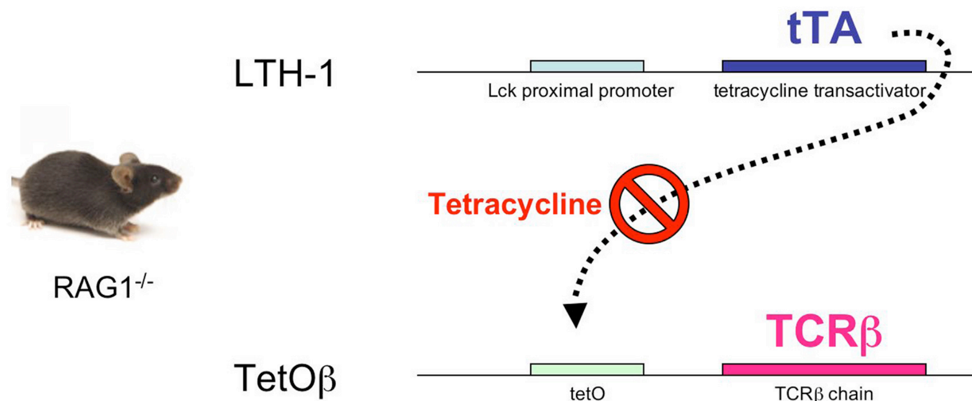


Figure 4.1: Mechanism of inducible TCRβ expression in TetOβ mice.

4.1.2.2 LckCre $Myc^{fl/fl}$

*Lck*Cre *$Myc^{fl/fl}$* were derived as detailed in the results section, and were also crossed onto the *$Rag2^{-/-}$* background to obtain *Lck*Cre *$Myc^{fl/fl}$* *$Rag2^{-/-}$* mice. Expression of the *Lck*Cre transgene (Wolfer et al., 2001) starts at the DN2 to DN3 stage transition in thymocytes. Therefore, c-Myc is conditionally deleted specifically at the DN3 stage in *Lck*Cre *$Myc^{fl/fl}$* and *Lck*Cre *$Myc^{fl/fl}$* *$Rag2^{-/-}$* mice.

4.1.2.3 Lck*Cre* $Ctnnb^{ex3}$

A mouse model for conditional stabilization of β-catenin, *$Ctnnb^{ex3}$* mice (Harada et al., 1999), was crossed with *Lck*Cre transgenic mice to produce *Lck*Cre *$Ctnnb^{ex3}$* compound mutant mice. These mice express a stabilized form of β-catenin starting at the DN3 stage of thymocyte development. β-catenin stabilization in this model is due to the abrogation of exon 3 of the *ctnnb* gene which encodes a GSK3-β phosphorylation site. Phosphorylation at this site marks

β-catenin for ubiquitination and subsequent degradation. Accordingly, in the absence of exon 3 a slightly shorter but stable form of β-catenin is expressed. *Lck*Cre *Ctnnb*^{ex3} mice were crossed with *Rag2*$^{-/-}$ mice to obtain *Lck*Cre *Ctnnb*^{ex3} *Rag2*$^{-/-}$ (Gounari et al., 2001).

4.1.2.4 Lck-*MyrAkt HA*

MyrAkt transgenice mice were a kind gift from Dr. Tsichlis and have been described (Malstrom et al., 2001). Briefly, C57BL/6 were engineered to express a transgene encoding an HA tagged Akt derivative under the *Lck*^{p56} promoter. MyrAkt was derived from Akt1 by introducing a myristoylation signal resulting in constitutive activation. Lck-MyrAkt HA mice therefore express constitutively active Akt1 in the thymus starting at the DN2 to DN3 transition.

4.1.2 Anti-CD3ε injection

Rag$^{-/-}$ mice express incomplete antigen receptor complexes consisting of CD3 subunits and the molecular chaperone calnexin at low levels on the cell surface. It is believed that the interaction of CD3ε with calnexin in the ER membrane masks the ER retention signals of calnexin and thus allows these complexes to escape to the outer cell membrane (Wiest et al., 1995). It has further been shown that injection of anti-CD3ε antibodies (α-CD3ε) into *Rag*$^{-/-}$ mice triggers signaling through these receptors and results in developmental progression similar to that observed in wt animals (Jacobs et al., 1994; Shinkai and Alt, 1994).
Anti-CD3ε antibody (50 µg/ mouse, clone 145-2C11, eBioscience) was injected i.p. into mice between 4 and 6 weeks of age. Animals were sacrificed at the indicated time points after injection for FACS analysis, cell sorting, or Western Blot.

4.1.3 Tetracycline treatment of animals

TetOβ-LTH *Rag1*$^{-/-}$ mice were fed tetracycline containing drinking water for 3- 4 weeks pre analysis (0.2 mg/ ml doxycycline and 20 g/ l sucrose in water) to

suppress TCRβ expression. The drinking water was changed every three days. Thymocytes were obtained and stained for FACS analysis.

Pregnant females were treated with tetracycline to suppress TCRβ expression in embryos. Upon birth intact thymic lobes were isolated from these suppressed mice and cultured on Transwell 0.4 µm filter cell culture plates (Costar), in thymic organ cultures in the absence of doxycycline to study the effect of preTCR induction.

Thymi from suppressed adult mice were isolated and passed through a 70 µm cell strainer to obtain single cell suspensions. The primary thymocytes were then cultured in the absence of doxycycline on OP9-DL1 stroma cells before sorting.

4.1.4 Preparation of embryonal thymi

Pregnant females were sacrificed on Day 16 of pregnancy and the uterus was obtained. Embryos were separated from maternal tissues and were kept on ice for 30 min. Thymic lobes were obtained with the help of a binocular stereo microscope (Zeiss) and passed through 5 ml FACS tubes equipped with filter mesh cap (BD) to obtain single cell suspensions. Thymi from neonatal mice were obtained similarly but were put in whole organ cultures.

4.2 Molecular Biology

4.2.1 Bacterial strains

E. coli strains DH5α and HB101 were used to amplify plasmid DNA.

4.2.2 Transformation and culture of *E. coli* cells

Chemically competent bacteria (obtained from the GRASP center core facility at Tufts) were thawed on ice. 100 µl of the suspension were incubated with 10 µl ligation reaction or 50 ng circular DNA for 10 min on ice. The cells were heat-shocked (30 sec, 42°C) and immediately chilled on ice. The volume was adjusted

to 600 µl with LB medium, and the suspension was incubated for 30 to 60 min (37°C, 230 rpm). Cells were then plated on selective medium and incubated at 37°C o/n. Cells were grown in LB medium (20 g/ l bacto-tryptone, 5 g/ l yeast extract, 5 g/ l NaCl). For solid media 10 g/ l agar were added. LB media were sterilized by autoclaving. For selective media the appropriate antibiotics were added after sterilization (usually ampicillin at 100 µg/ ml).
XL10-Gold Ultracompetent cells (Stratagene) were used for transformation of ligation reactions. Transformation was carried out as recommended by the manufacturer.

4.2.3. Plasmid purification

Plasmids were purified using Mini- and Maxiprep kits purchased from Qiagen according to the manufacturer's recommendations. CsCl preparations for cell culture purposes were made by the GRASP center core facility at Tufts.

4.2.4 Detection of DNA on agarose gels

DNA was analyzed on and/ or purified from 1.5% agarose gels (1.5% (w/v) agarose in TAE buffer) containing 10 ng/ ml ethidium bromide. For size comparison DNA Ladder (100 bp, 1 kb) purchased from NEB was used. Electrophoresis was carried out in TAE buffer at 130- 150 V.

TAE buffer (50x)

242 g	Tris-base.
57.1 ml	glacial acetic acid
18.6 g	EDTA

Adjust volume to 1l with distilled H_2O.

DNA loading buffer (10x)

0.25% (w/v)	Orange G (Sigma)
10% (v/v)	glycerol in water

4.2.5 Restriction digest

Restriction digests were carried out using NEB restriction enzymes and supplementary 10x reaction buffers. The reaction volume was chosen in a way that the (v/v)% of restriction enzyme was equal or less than 10%. 5 U of enzyme were used per µg DNA and the digest was performed for 1h at 37°C.

4.2.5.1 Cloning of pIRES_TetOβ_Neo

The pIRES2-EGFP vector (Clontech) was sequentially digested with *Ase*I and *Hpa*I and blunted with Klenow polymerase (1 U) to obtain a 3.3 kb fragment devoid of the CMV promoter, the IRES, and the EGFP but containing the neomycin resistance cassette. This fragment was used as the backbone for pIRES_TetOβ_Neo. A blunted 2.9 kb *Xho*I fragment (the same one that was used to generate TetOβ transgenic mice) containing 7 tetO sequences, a minimal CMV promoter, a rat β-globin intron, a TCRβ cDNA and a rat β-globin polyadenylation signal, was then ligated into the backbone to obtain pIRES_TetOβ_Neo.

4.2.6 Genomic DNA extraction from tail biopsies

Tail clippings were obtained from 2.5 week old mice. Approximately 2 mm of tail were cut and digested at 56°C o/n in 750 µl tail extraction buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1 % SDS, pH 8) with proteinase K (Roche) at a final concentration of 0.5 mg/ml. 250 µl saturated NaCl solution were added, and the sample was thoroughly mixed. The salt precipitate was collected in a table top centrifuge at full speed for 10 min and 750 µl of the supernatant were transferred to a new tube for isopropanol precipitation (500 µl). DNA precipitate was collected at maximum speed for 20 min. Pellets were washed once with 70% ice cold ethanol, air dried, and resuspended in 500 µl water for PCR analysis.

4.2.7 Genomic DNA extraction from single cell suspensions

Approximately 2x 10^5 cells were digested in 100 µl of 1x PCR reaction buffer/ 0.5% Triton X-100/ 200 µg/ ml proteinase K for 1 hour at 60°C followed by heat inactivation of proteinase K for 15 min at 95°C. 1 µl of this mixture was used for PCR.

4.2.8 PCR based genotyping

PCR screening was performed using RedStar Taq polymerase (Sigma) and genomic DNA prepared from tail biopsies of mice. Primer pairs for genotyping and deletion PCRs were as follows:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
*Lck*Cre	ATCGCTCGACCAGTTTAGT	CGATGCAACGAGTGATGA
$Myc^{fl/fl}$	GCCCCTGAATTGCTAGGAAGACTG	CCGACCGGGTCCGAGTCCCTATT
$Ctnnb^{ex3}$	TAGAAGGTTCTTGAGGTAGTGGTC	AAACTCCTCTTCAGACCTAATAAC
LTH1	AGCTGCTTAATGAGGTCGGA	GCTTGTCGTAATAATGGCGG
TetOβ	AGACGCCATCCACGCTGTTTTGA	AGAGGTACAGTGCTGAGTCTCCT

The MyrAkt transgene was detected with three primers amplifying a 240 bp fragment representing endogenous sequence and a 422 bp fragment indicating the presence of the transgene. The primers were as follows:
PKBe3 5'-TGGGGCTTGAAAGGTGGGCTCA-3'
PKBe5 5'-ATGCTGGACAAGGACGGGCACA-3'
PKBi5 5'-AGAACGGAGTTCCCACTGCCTG-3'

A typical PCR reaction contained the following:

1µl	template DNA from tail prep
3µl	10x reaction buffer
3µl	dNTP mix (2 mM)
0.2µl	primer1 (50 µMl)
0.2µl	primer2 (50 µM)
1µl	RedStar Taq polymerase (5 U)
21.6µl	H_2O

4.2.9 Semi-quantitative PCR

mRNA was extracted from sorted cells using the High Pure RNA Isolation Kit (Roche). cDNA from 50,000 cells was prepared with the Superscript-II RT kit (Invitrogen). Samples were equilibrated with respect to β-Actin using SYBR-green quantitative-PCR on an OpticonII machine (BioRad). Semi-quantitative-PCR was performed on 1:5 serial dilutions. All PCR amplifications were performed in a volume of 30 µl, and used touchdown conditions (increments of –1°C per cycle for the first five annealing steps starting from 62°C, followed by 30 cycles with an annealing temperature of 56°C). Primer pair sequences were as follows:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
p53	CCCGAGTATCTGGAAGACAG	ATAGGTCGGCGGTTCAT
Bcl-x$_L$	AGCAACCGGGAGCTGGTGGTCGAC	GACTGAAGAGTGAGCCCAGCAGA
Tcrb	AGCTGAGCTGGTGGGTGAATGG	CCTCTGGCCACTTGTCCTCCTCTG
Ptcra	GGCACCCCCTTTCCGTCTCT	TTTGAAGAGGAGCAGGCGCA
Myc	TCACCAACAGGAACTATGAC	AAGCTCTGGTTCACCATGTC
Nmyc1	GATGATCTGCAAGAACCCAG	GGATGACCGGATTAGGAGTG
Ccnd2	CTTCCAAGCTGAAAGAGACC	TACCCAACACTACCAGTTCC

Ccnd3	CGAGCCTCCTACTTCCAGTG	GGACAGGTAGCGATCCAGGT
Ccne1	TCCTGGCTGAATGTCTA	CTTCTCTATGTCGCACCA
Actinb	TGGAATCCTGTGGCATCCATG	TAAAACGCAGCTCAGTAACAG

4.2.10 Quantitative real-time PCR

Quantitative RT-PCR was performed using an ABI7300 machine (Applied Biosystems). $p21^{Cip1}$ and Gadd45α were determined relative to GAPDH expression using TaqMan Gene Expression Assays from Applied Biosystems. Tis21 and Id3 were assayed with SYBR Green technology and expression levels were determined relative to β-Actin. Primer sequences were as follows (forward, reverse):

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Actinb	ATGGTGGGAATGGGTCAGAA	TCTCCATGTCGTCCCAGTTG
Tis21	ACGCACTGACCGATCATTACA	GGCTGGCTGAGTCCAATCTGG
Id3	GGCACTGTTTGCTGCTTTAGG	GTAGCAGTGGTTCATGTCGTC

All qRT-PCR reactions were analyzed in triplicate in a 20 µl reaction containing 0.3 µM of each primer. The conditions for all qRT-PCRs were: 50°C for 2 min, 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

C_T values were determined using 7300 sequence detection software version 1.2 for analysis, and data were exported to Micorsoft Excel. Data were then analyzed and evaluated according to the relative $\Delta\Delta C_T$ method. Each sample C_T mean was calculated and standard deviations (s) were calculated for each mean C_T value. Briefly, the amount of target gene, normalized to an endogenous reference (β-Actin or Gapdh in this case) and relative to a calibrator (in this case changes were expressed relative to the expression levels in wt DN3 cells), is given by:

$$2^{-\Delta\Delta C_T}$$

where

$$\Delta C_T = C_{T\ target} - C_{T\ reference}$$

and the standard deviation of ΔC_T is

$$s = (s_{target}{}^2 + s_{reference}{}^2)^{1/2}$$

$\Delta\Delta C_T$ was then calculated according to

$$\Delta\Delta C_T = \Delta\Delta C_{T\ test\ sample} - \Delta\Delta C_{T\ calibrator\ sample}\ .$$

The standard deviation of $\Delta\Delta C_T$ is that of the DCT of the test sample and was used to calculate the range (upper and lower limits) of the fold difference of expression (depicted as error bars in the graphs).

4.2.11 Microarray analyses

Pools of five *Lck*Cre *Myc*$^{fl/fl}$ *Rag*$2^{-/-}$ and *Lck*Cre *Rag*$2^{-/-}$ control mice were injected with 50 µg of α-CD3ε antibodies or PBS to induce preTCR signaling. Animals were sacrificed 24 and 48 hours after injection and DN3 and DN4 cells were sorted for RNA preparations. RNA was obtained using the RNeasy Micro kit (Qiagen) and was sent to a commercial service provider (Cogenics) for further processing. The service included linear amplification of 50 ng RNA from each sample and labeling with Cy3 and Cy5 fluorophores. Samples were hybridized onto Agilent 4122F mouse whole genome gene chips using a dye reversal strategy to control for experimental variation. Raw data were then analyzed to obtain lists of genes that changed upon preTCR signaling in the absence and presence of c-Myc mediated proliferation and returned as Microsoft Excel file for further analysis by us.

4.3 Cell Biology

4.3.1 Mammalian cell lines

293T	human embryonic kidney cell line used for retrovirus production
NIH 3T3	mouse fibroblast cell line used for viral titration
SciET27F	preTCR deficient thymocyte cell line, derived from a spontaneously arising thymoma in Scid mice. These cells express pTα but no TCRβ chains.
SCB.29	preTCR competent thymocyte cell line, derived from SciET27F via stable transfection with a construct encoding a productively rearranged TCRβ chain composed of Jβ2.3Vβ8.2 (Groettrup et al., 1992)
OP9-DL1	BM derived stroma cell line expressing DL1 for *ex vivo* coculture (Schmitt and Zuniga-Pflucker, 2002)
OP9-GFP	BM derived stroma cell line expressing GFP for *ex vivo* coculture (Schmitt and Zuniga-Pflucker, 2002)

4.3.2 Transfection

4.3.2.1 Calcium phosphate transfection for retrovirus production

293T cells were cultured in IMDM/ 10% FCS/ Pen/Strep at 37°C and 5% CO_2. 24 h prior to transfection 0.5x 10^6 cells were plated on 6 cm dishes. Medium was changed three hours prior to transfection. 2x BES was thawed at RT and could not be refrozen. $CaCl_2$/ BES/ DNA precipitate was prepared as follows:

For each 6 cm dish a solution of 8 µg retroviral DNA, 6 µg of pMD. MLV gag.pol (helper plasmid), 2 µg of pMD.G (VSV-G pseudotype plasmid), 31 µl of 2M $CaCl_2$ and enough water to bring the total volume to 0.25 ml was prepared. To this mixture 0.25 ml 2x BES were added drop-wise, while air was blown into the solution using a pipetting aid equipped with a disposable Pasteur pipet. The transfection mix was incubated at RT for 20 min and added to the cell culture medium, trying to cover as much of the surface as possible. 12 to 16 h after transfection the medium was exchanged, and supernatants were harvested 24, 48, and 72 h later. Retroviral titers were determined by infecting NIH 3T3 fibroblasts and viral supernatants were concentrated by ultracentrifugation at 16,500 rpm using Sorvall rotor SW-41T1 for 2- 3 hours. Aliquots at 10^7 infectious particles per ml were stored at –80°C until needed.

2M $CaCl_2$

87.6 g $CaCl_2 \cdot 6H_2O$

were dissolved in a final volume of 200 ml distilled water. The solution was filter-sterilized and stored at 4°C.

2X BES

50	mM	BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid)
280	mM	NaCl
1.5	mM	$Na_2HPO_4 \cdot 7H_2O$

pH was adjusted to 6.9 with HCl. Aliquots were stored at –20°C.

4.3.2.2 Transfection of SciET27F cells

For electroporation SciET27F cells were maintained at densities lower than 10^6 cells/ ml and were diluted to 3x 10^5 cells/ ml the night before transfection. On the day of transfection 1x 10^7 cells were resuspended in 450 µl of IMDM/ 20% FBS and transferred into a 0.4 cm electroporation cuvette (Invitrogen). 20 µg of pIRES_TetOβ_Neo plasmid were added, and the cell suspension was mixed. The cuvette was incubated at RT for 10 min. Immediately before application of

the pulse, the suspension was mixed by gently flicking the cuvette. Transfection was carried out at 260 V, 960 µF with a time constant between 22- 24 msec. The cuvette was then incubated at RT for an additional 15 min before cells were transferred into a flask containing 10 ml of IMDM/ 20% FBS. Next day the number of live cells was estimated using trypane blue staining, and cells were plated on 96 well plates at a concentration of 1000 cells per well in 200 µl IMDM/ 10% FBS/ 1.4 mg/ ml G418. Selection pressure with 1.4 mg/ ml G418 was maintained for 3- 4 weeks until G418 resistent cells were visibly growing in the plates. These cells were pooled from different wells and expanded in 8 pools, all of which were examined by PCR with respect to the presence of the TetOβ construct (primers as for genotyping mice, see above).

4.3.3 Retroviral spin infection of TetOβ positive clones

TetOβ positive clones were infected with a retrovirus (MigR1-rtTA, a gift from Dr. Philip Tsichlis) encoding for a reverse tetracycline transactivator (rtTA) and EGFP. As opposed to the situation in the inducible mice described above, TCRβ expression is induced by tetracycline in this system, because the rtTA is inactive in the absence but active in the presence of tetracycline.

For spin infections 10^5 cells were spun down in a 6 well plate and supernatants were replaced with 2 ml of IMDM/ 10% FBS/ 8 µg/ ml polybrene containing ~10^6 viral particles. The 6 well plates were centrifuged for 1.5 hours at 2200 rpm and further incubated for 4- 5 hours before the medium was changed back to IMDM/ 10% FBS/ 1.4 mg/ ml G418. 48 h later $EGFP^+$ cells were sorted and the SciTetOβ cell line was obtained from an arbitrarily chosen, expanded single cell.

4.3.4 OP9-DL1 coculture

4.3.4.1 Primary cells

OP9-DL1 coculture was established several years ago as a novel culture system that allows *ex vivo* T cell differentiation of primary thymocytes (Schmitt and Zuniga-Pflucker, 2002) (and bone marrow cells). OP9-GFP and OP9-DL1 cell lines were a generous gift from Dr. Juan Carlos Zuñiga-Pflücker. OP9 cell lines were maintained in α-MEM/ 20%FBS/ 10 U/ ml penicillin and streptomycin. 2x 10^4 OP9 cells per well were plated the day before coculture in 24-well plates. 5x 10^4 sorted thymocytes were added to the stroma cells next day, and cocultures were maintained in the presence of 5 ng/ml Flt3L (Peprotech) and 1 ng/ml IL-7 (R&D Systems) for 4 to 6 days before they were harvested for FACS analysis. For inhibitor studies γ–secretase inhibitor X (GSI X, Calbiochem) was dissolved in DMSO and added at a final concentration of 1 μM to the cell culture medium (final concentration of 0.1 % DMSO in the medium). Control wells were treated accordingly with 0.1 % DMSO. Significant numbers of DP cells could generally be observed around Day 3 of coculture when starting from DN4, and around Day 5 when starting from DN3 stage, adult, murine thymocytes.

4.3.4.2 SciET27F and SCB.29 coculture on OP9 stromal cells

SciET27F and SCB.29 cells were maintained in IMDM/ 10% FBS/ 0.1% 2-mercaptoethanol. For the coculture experiment cells were plated on confluent layers of OP9-GFP and OP9-DL1 in 6-well plates at a concentration of 1x 10^6/ ml. Cells were cocultured for 16, 5, 2, 1, and 0 hours before they were harvested for further experiments. Live, non-stroma cells were sorted, lysed, and an amount of lysate corresponding to 2x 10^6 cells was loaded per lane of a NuPage Bis-Tris gradient gel. Western blot for p-Akt and Akt was performed as detailed below.

4.3.5 CFSE labeling of primary thymocytes

Sorted cells were re-suspended in 1 ml PBS/ 0.1% BSA at a concentration of 1-5x 10^6 cells/ ml and incubated with 1 µM 5,6-carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) at 37 °C for 10 min before washing extensively with α-MEM/ 20% FBS. Viability after labeling exceeded 60%. CFSE labeled cells were cultured on OP9-DL1 or OP9-GFP stroma cells in α-MEM/ 20% FBS.

4.3.6 Flow Cytometry and Antibodies

4.3.6.1 Surface stainings

Four-color-FACS stainings were performed for analysis and cell sorting of primary and cultured thymocytes from mice. Usually, 0.5- 1 x10^6 cells were stained in a total volume of 50- 200 µl FACS buffer (PBS/ 2% FBS). Antibodies were from BD PharMingen or eBioscience: anti-CD3ε-phycoerythrin-(PE), –biotin (17A2 and 500A2), anti-B220-CyChrome (RA3.6B2), anti-CD4-fluorescein-5-isothiocyanate-(FITC), -CyChrome, -PE, -Allophycocyanin-(APC), anti-CD8-FITC, -CyChrome, -PE, -APC (53.6.7), anti-TCRβ-PE, -CyChrome (H57), anti-TCRγδ-PE, -biotin (GL3), anti-pan-NK-PE, -biotin (DX5) anti-CD44-FITC, -PE (IM7), anti-CD25-APC (PC61), anti- Gr1-PE, -biotin (RB6.782), anti-CD11b-PE, -biotin (M1/70), anti Ter119-PE, -biotin. Biotinylated antibodies were detected with Streptavidin-PE, -CyChrome or -APC. If DN thymocytes were to be analyzed mature cells expressing lineage (lin) markers (CD4, CD8, TCRβ, TCRγδ, CD19, Gr1, Mac1, Ter119 and DX5) were excluded by electronic gating. FACS analysis was performed on a Cyan flow cytometer (DakoCytomation) and data were analyzed using FlowJo software (Tree Star).

4.3.6.2 Intracellular FACS staining

Intracellular staining was performed after surface staining was completed. Cells were permeabilized with 0.04% saponin containing FACS buffer

(permeabilization buffer) in the presence of TCRβ or β-catenin antibodies for 1 hour on ice. Samples were washed twice with 1 mL of permeabilization buffer before FACS analysis. Intracellular Bcl-2 was analyzed using anti–murine Bcl-2 (mAb 3F11, BD PharMingen) and purified hamster IgG (anti-trinitrophenol, PharMingen) as an isotype control. 1x 10^6 cells were incubated with 20 µl Bcl-2-PE assay solution (BD PharMingen) and incubated on ice in the dark for 1 hour. Samples were washed with permeabilization buffer. FACS analysis was performed on a Cyan flow cytometer (DakoCytomation) and data were analyzed using FlowJo software (Tree Star).

4.3.6.3 Detection of apoptotic cells and cell cycle analysis

Apoptotic cells exhibit an increased number of phosphatidylserine molecules on the outer cell membrane. Annexin V binds to phosphatidylserine (more strongly than to other phospholipids), and an increase in Annexin V staining can therefore be used to discriminate apoptotic cells. Annexin V-FITC labeling kit (BD PharMingen) was used according to the manufacturer's instructions. Briefly, FACS surface stainings were performed as detailed above. 1x 10^5 cells were then resuspended in 100 µl Annexin V binding buffer and 5 µl Annexin V-FITC were added shortly before data acquisition using a FACSCalibur (BD).

Cell cycle analysis was performed using the DNA intercalating dye 7-Aminoactinomycin D (7-AAD), which can be detected by flow cytometry. 1x 10^6 primary thymocytes were subjected to surface staining as detailed above and were then incubated in 100 µl of permeabilization buffer (see Intracellular FACS staining procedure) plus 20 µl 7-AAD (BD Bioscience) for 2 hours in the dark on ice. Samples were washed in permeabilization buffer and subjected to FACS analysis.

4.3.6.4 Enrichment of DN cells by bead depletion

For cell sorting of DN populations, DN thymocytes were enriched by depletion of lin^+ cells using Streptavidin conjugated magnetic beads (Dynal). Cells were

stained for lineage markers using biotinylated antibodies in FACS buffer for 10 min on ice. The staining volume was approximately 300 µl per thymus. Cells were then washed with FACS buffer two times and incubated with Streptavidin magnetic beads according to the manufacturer's recommendations for 15 min on ice. Samples were cleared using an MPC-L magnet (Dynal), and the enriched DN fractions were subjected to further staining and analysis.

4.3.6.5 Cell sorting

Cell sorting was performed on a MoFlo cell sorter (DakoCytomation) at the Tufts Laser Cytometry core facility. Cells were collected in 15 ml tubes containing several ml of α-MEM/ 20% FBS.

4.3.7 Western Blot

Pellets of total thymocytes were lysed in RIPA buffer (50 mM Tris-HCl/ 150 mM NaCl/ 1% NP-40/ 1% Sodium Desoxycholate/ 1 mM EDTA/ 0.1% SDS, pH 7.4) supplemented with Complete Mini protease inhibitor cocktail (Roche) and 1 mM PMSF. Samples were resolved on NuPage Bis-Tris gradient gels (Invitrogen) and transferred onto nitrocellulose or Polyvinylidene Fluoride (PVDF) membranes using the Sure Lock Mini cells according to the manufacturer's instructions (Invitrogen). Non-specific binding was blocked in blocking buffer (5% non-fat milk in PBS/ 0.1% Tween-20), followed by incubation with the primary antibodies and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocking buffer. Western blots were probed with mouse anti-β-catenin (clone 14, BD Transduction Laboratories, 1: 2000), rabbit anti-Myc (Cat.# 06-340, upstate cell signaling solutions, 1: 1000), mouse anti-p27 (F-8, Santa Cruz Biotechnology, 1: 2000) rabbit anti-Cyclin D2 (M-20)/ D3 (C-16) (Santa Cruz Biotechnology, 1: 3000), rabbit anti-Phopho-Akt (Thr308) and rabbit anti-Akt (Cell Signaling Technologies, 1: 1000 in 5% BSA/ PBST), and rabbit anti-GAPDH from (Abcam, 1: 2000). Secondary HRP coupled goat anti-rabbit IgG (Santa Cruz) and

goat anti-mouse IgG (BioRad) antibodies were used 1: 15000. The signal was detected using the enhanced chemiluminescence Plus (ECL-Plus kit, GE-Healthcare).

5 Results

5.1 The role of c-Myc mediated proliferation at the preTCR checkpoint

c-Myc has an undisputed role in cell proliferation. Based on the fact that the preTCR induces a wave of proliferation and earlier evidence indicating that c-Myc may play a role in T-cell development we designed experiments to characterize its function at the β-selection checkpoint.

5.1.1 PreTCR signaling induces c-Myc expression

5.1.1.1 preTCR induction in TetOβ-LTH Rag1$^{-/-}$ *mice*

Initially, it was of interest to establish whether c-Myc expression changes at the β-selection checkpoint. Therefore, we examined a novel mouse strain that allows inducible preTCR expression. More specifically, these mice carry a transgene encoding a TCRβ chain under the control of a minimal CMV promoter augmented with seven tet operator (tetO) sequences. TCRβ expression is dependent on a second transgene that results in expression of the TetR-VP16 transactivator (tTA) in immature thymocytes under the control of the *Lck* gene proximal promoter (LTH-1) (Labrecque et al., 2001). Expression of the transgenic TCRβ chain in compound TetOβ-LTH mice is suppressed in the presence and induced in the absence of tetracycline (see **Figure 4.1** in "Materials and Methods"). To avoid simultaneous expression of multiple T-cell receptors, TetOβ-LTH mice have been crossed onto the *Rag1*$^{-/-}$ background because *Rag1*$^{-/-}$ mice lack the capacity to rearrange their TCR chains (and are therefore developmentally arrested at the CD44$^-$CD25$^+$DN3 stage).

Indeed, the thymic profile of TetOβ-LTH *Rag1*$^{-/-}$ mice treated with tetracycline resembled that of *Rag1*$^{-/-}$ mice with a thymic cellularity of 2- 3x 10^6 DN thymocytes, indicating that the drug effectively suppressed TCRβ transgene expression and prohibited preTCR assembly (data not shown). In the absence of tetracycline tTA induced the expression of the transgenic TCRβ chain, leading to

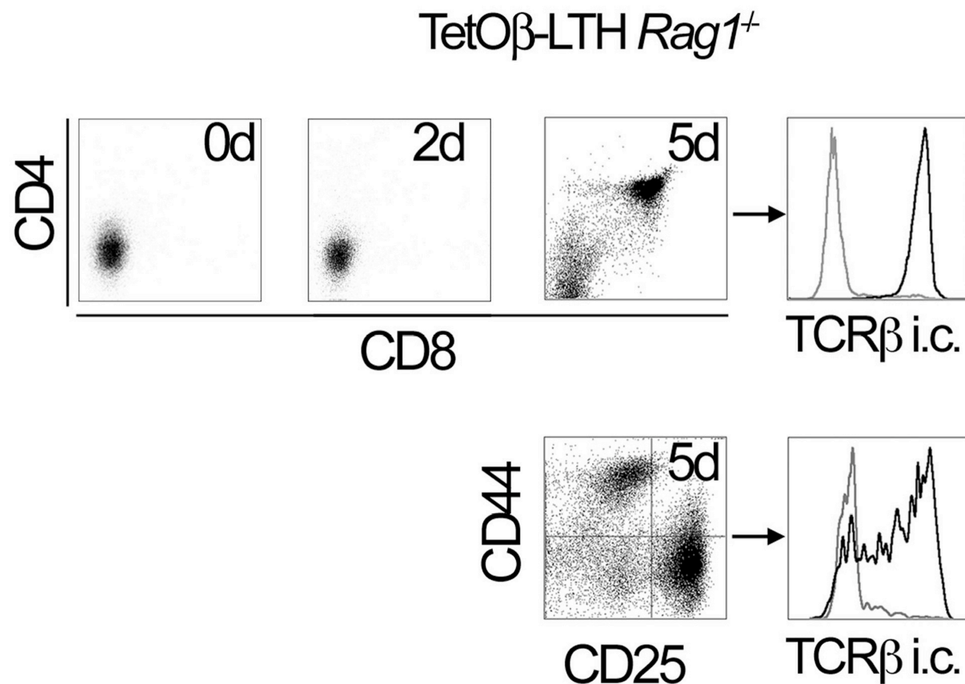


Figure 5.1: Developmental progression in TetOβ-LTH *Rag1*$^{-/-}$ thymocytes. FACS analysis of thymocytes from neonatal thymic organ cultures derived from mice that had been treated with tetracycline during gestation. Thymic lobes were kept in organ cultures in the absence of tetracycline for the indicated times. Upper dot plots show CD4 versus CD8 and lower dot plot show CD44 versus CD25 surface profiles of gated lin$^-$ cells. Histograms show intracellular TCRβ expression in the DP (dark grey) versus DN (light grey) and the DN4 (dark grey) versus DN3 (light grey) subsets.

the assembly of a functional preTCR and developmental progression to the DP stage. Untreated thymi contained 75- 80% DP cells and had a thymic cellularity of 50- 60x 10^6 cells (data not shown). Thymic lobes isolated from newborn mice treated with tetracycline during gestation were placed in organ cultures for five days without tetracycline. This resulted in developmental progression, with over 70% of the thymocytes reaching the DP stage and a greater than 10-fold increase in the cellularity of the lobes. All DP cells and the majority of CD44$^-$ CD25$^-$ DN4 cells expressed intracellular TCRβ chains (**Figure 5.1**) indicating that

they were able to assemble a functional preTCR and pass the β-selection checkpoint. Thus, these newly developing thymocytes represent a good model system for assessing the downstream of effects of preTCR signaling.

5.1.1.2 c-Myc induction in TetOβ-LTHCre Rag1$^{-/-}$ thymocytes

The effect of preTCR signaling on c-Myc was examined after induction of TCRβ expression in TetOβ-LTH *Rag1*$^{-/-}$ thymocytes. To this end TetOβ-LTH *Rag1*$^{-/-}$ and LTH *Rag1*$^{-/-}$ control animals that express only the LTH transgene but not the TetOβ construct, and are therefore unable to induce TCRβ expression, were treated with tetracycline. Thymocytes were isolated and cocultured on OP9-DL1 stromal cells for 24 hours in tetracycline-free culture medium. Suspension cells were recovered from the cocultures and thymocytes (Thy-1 positive cells) were sorted. Whole cell lysates prepared from the sorted cells were used in Western blots to determine the levels of c-Myc protein. 24 hours after induction of preTCR signaling TetOβ-LTH *Rag1*$^{-/-}$ cells showed an accumulation of c-Myc protein compared to LTH *Rag1*$^{-/-}$ control cells (**Figure 5.2**) indicating that c-Myc was rapidly induced in response to preTCR signals. Similar blots detected no changes in the levels of cyclin D2 and D3 24 hours after induction of preTCR signaling (data not shown).

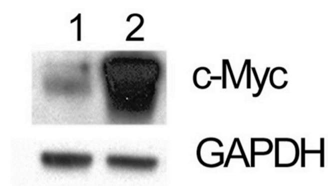


Figure 5.2: c-Myc induction in TetOβ-LTH *Rag1*$^{-/-}$ thymocytes. c-Myc Western blot of thymocytes from LTH *Rag1*$^{-/-}$ and TetOβ-LTH *Rag1*$^{-/-}$ mice that had been treated with tetracycline prior to coculturing with OP9-DL1 cells in tetracycline free growth medium for 24 hours. Thy1^{+} cells were sorted from the cocultures and total cell lysates were used for Western blotting.
Lane 1: LTH *Rag1*$^{-/-}$, lane 2: TetOβ-LTH *Rag1*$^{-/-}$

5.1.1.3 c-Myc induction upon preTCR signals in Rag$^{-/-}$ mice

In order to confirm the data obtained in the inducible mouse model, we sought to analyze a different model system. Therefore, c-Myc expression was further examined after induction of preTCR like signaling in Rag deficient mice by α-CD3ε treatment. Injection of the α-CD3ε mAb (2C11) in Rag deficient mice has been previously shown to mimic preTCR signaling and promote developmental progression of DN3 cells to the DP stage (Jacobs et al., 1994; Shinkai and Alt, 1994).

To determine the effect of these signals on c-Myc expression, *Rag2*$^{-/-}$ thymocytes were isolated 0, 6, 16, and 48 h post i. p. injection of purified α-CD3ε mAb (50 µg/ mouse). Protein lysates were prepared for Western blot analyses. c-Myc protein levels increased gradually throughout the period of observation (48h),

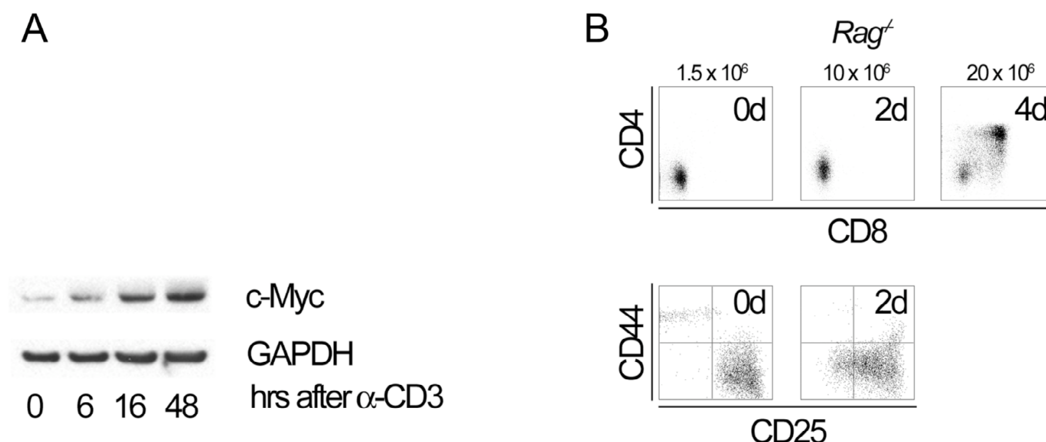


Figure 5.3: c-Myc induction upon α-CD3ε treatment in *Rag*$^{-/-}$ thymocytes. (A) c-Myc Western blot from *Rag*$^{-/-}$ mice that were injected with 50 µg of α-CD3ε mAb at the indicated time points prior to sacrifice. Total thymic lysates were obtained for Western blotting. (**B)** FACS analysis of α-CD3ε mAb induced thymocyte development in *Rag*$^{-/-}$ mice. Dot plots show thymocyte expression profiles for CD4 versus CD8 (top) and CD44 versus CD25 (bottom, data gated on lin^{-} cells) from *Rag*$^{-/-}$ mice 0, 2, and 4 days after i. p. injection of α-CD3ε antibody.

starting at 6h after α-CD3ε injection **(Figure 5.3A**). To correlate the c-Myc protein levels with the course of the developmental progression induced by α-CD3ε treatment, we stained thymocyte suspensions of similarly treated mice with antibodies against CD4 and CD8, or against lineage markers combined with CD44 and CD25, followed by FACS analysis (**Figure 5.3B**). 48h after α-CD3ε injection thymocytes had not yet developed to the DP stage and were still undergoing the transition from the DN3 to the DN4 stage. Thus, c-Myc induction preceded developmental progression indicating that it was a consequence of preTCR signaling and not a result of the developmental transition.

Taken together, these data suggest that c-Myc expression is induced by preTCR signaling and emphasize the need for detailed analysis to determine its role at the preTCR dependent stages of thymocyte development.

5.1.2 Abnormal thymocyte development upon conditional c-Myc ablation in mice

5.1.2.1 Generation of LckCre Myc$^{fl/fl}$ *mice*

As pointed out in the introduction, Myc deficiency leads to developmental defects in BM progenitors. To assess the impact of c-Myc specifically downstream of the preTCR it was therefore necessary to avoid these earlier developmental defects. Hence, a novel mouse model that allows conditional ablation of c-Myc starting at the DN3 stage of thymocyte development was generated (**Figure 5.4**). This was achieved by crossing *Myc*$^{fl/fl}$ mice (de Alboran et al., 2001) that carry LoxP sites flanking the coding exons 2 and 3 of the *Myc* gene with mice expressing Cre under the control of the proximal p56^{Lck} promoter (*Lck*Cre) (Lee et al., 2001).

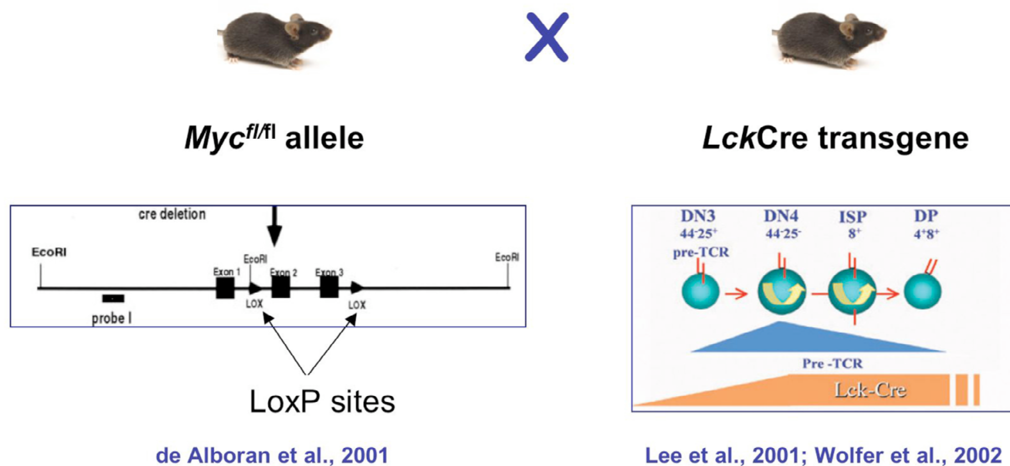


Figure 5.4: The *Lck*Cre *Myc*$^{fl/fl}$ mouse model. Conditional ablation of c-Myc at the β-selection checkpoint was achieved by combining *Myc*$^{fl/fl}$ with *Lck*Cre transgenic mice. *Lck*Cre expression starts at the DN2 to DN3 transition. Upon Cre expression exons 2 and 3 of the *Myc* gene are excised, effectively abolishing c-Myc expression.

Cre mediated deletion of exons 2 and 3 of the *Myc* gene in compound mutant *Lck*Cre *Myc*$^{fl/fl}$ mice was detectable at the DN3 stage (data not shown) and thus ablation of c-Myc was expected to coincide with the onset of preTCR signaling. Efficiency and stage specificity of Cre mediated ablation of c-Myc in these mice was examined by semi-quantitative RT-PCR, using cDNA derived from sorted DN3 and DN4 stage thymocytes. Expression of c-Myc was reduced about 5-fold at the DN3 stage and was completely abrogated at the DN4 stage (**Figure 5.5A**). Western blot analysis of extracts from similarly sorted cells showed that *Lck*Cre control thymocytes had lower levels of c-Myc protein at the DN3 stage than at the preTCR dependent DN4 stage (**Figure 5.5B**). Deletion of the *Myc* gene in *Lck*Cre *Myc*$^{fl/fl}$ thymocytes severely diminished the expression of c-Myc protein both in the DN3 and DN4 subsets.

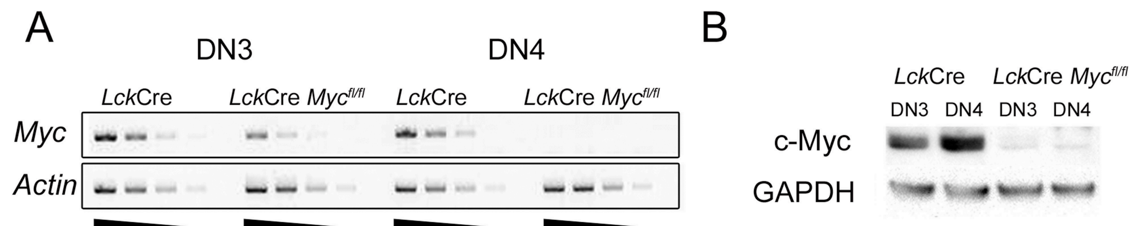


Figure 5.5: Characterization of *Lck*Cre *Myc*$^{fl/fl}$ mice. (**A, B**) Efficiency of c-Myc ablation at the DN3 and DN4 stages of thymocyte development. Semi-quantitative c–Myc RT-PCR with 5-fold serial dilutions (**A**) and c-Myc Western blots (**B**) were performed on FACS sorted cells from the indicated mice and subsets.

Thus, a novel model system has been generated that allows examination of the developmental impact of c-Myc deficiency specifically at the β-selection checkpoint.

5.1.2.2 Phenotype of LckCre Myc$^{fl/fl}$ mice

To determine the phenotypic impact of c-Myc ablation on thymocyte development we compared the thymocyte subset distribution (**Figure 5.6A**) and thymic cellularity (**Figure 5.6B**) of *Lck*Cre *Myc*$^{fl/fl}$ and control embryos and adult (5-8 weeks old) mice. Adult *Lck*Cre *Myc*$^{fl/fl}$ contained ~10 times fewer thymocytes ($1.2x10^7$±$0.11x10^7$) than *Lck*Cre controls ($8.9x10^7$±$0.66x10^7$). This mainly reflected a ~30-fold reduction in the number of $CD4^+CD8^+$ DPs in *Lck*Cre *Myc*$^{fl/fl}$ ($2.7x10^6$±$0.17x10^6$) compared with the equivalent *Lck*Cre control cells (8.4 $x10^7$±$0.92x10^7$) (**Figure 5.6B**), and the reduction of the subsequent SP stages. Cellularity at the DN3 stage of *Lck*Cre Myc$^{fl/fl}$ mice was elevated with respect to *Lck*Cre controls (P=0.02, **Figure 5.6B**). Interestingly, the number of *Lck*Cre

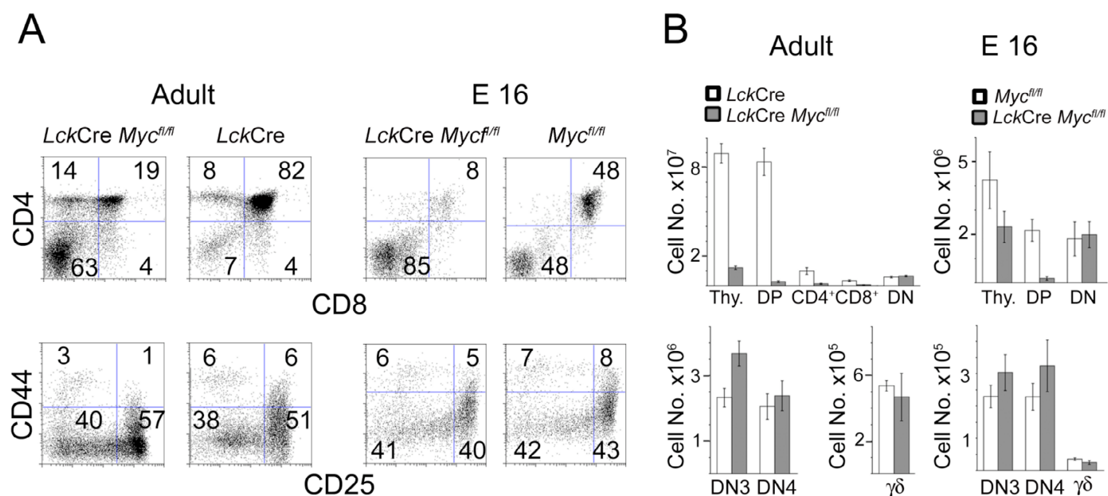


Figure 5.6: c-Myc ablation at the DN3 stage impacts thymic cellularity and subset distribution. (**A)** FACS analyses for CD4/CD8 (top) and CD44/CD25 (bottom, gated on lin$^-$ events) surface expression in *Lck*Cre *Myc*$^{fl/fl}$ and *Lck*Cre (Adult= 5- 8 weeks) or *Myc*$^{fl/fl}$ (E16= Embryonic day 16) mice. Numbers given indicate the subset distribution, i.e. the percentage of cells in the indicated quadrant. (**B**) Cellularity was determined by multiplying the number of total thymocytes with the percentages from **A** (for DN3, DN4 also considering the percentage of lin$^-$ cells). Error bars indicate S.D. *Thy.*: Total number of thymocytes, γδ: TCRγδ$^+$ thymocytes. Numbers of adult animals analyzed to obtain these statistics were as follows ($N_{LckCre\ Mycfl/fl}$, N_{LckCre}): total thymocytes (N=27, N=11), DP/ $CD4^+$/ $CD8^+$/ DN (N=13, N=6), DN3/ DN4 (N=9, N=7), TCRγδ$^+$ thymocytes (N=10, N=5). Embryo data are based on 3 *Myc*$^{fl/fl}$ control embryos and 11 *Lck*Cre *Myc*$^{fl/fl}$.

Myc$^{fl/fl}$ DN4 stage thymocytes was comparable to that of *Lck*Cre controls indicating that development through the preTCR checkpoint was not inhibited.
Similar results were obtained from embryonic thymi (E16) (**Figure 5.6A, B**). Control *Myc*$^{fl/fl}$ embryonic thymi contained on average $4.2x10^6$±$1.2x10^6$ cells. This number was reduced by ~50% in *Lck*Cre Myc$^{fl/fl}$ mice ($2.3x10^6$±$0.6x10^6$), accounting for a ~10-fold decrease in the number of DPs ($2.2x10^6$±$0.4x10^6$ versus $1.9x10^5$±$0.8x10^5$). The DN compartment remained unaltered in profile (**Figure 5.6A**) and cellularity (**Figure 5.6B**).
Collectively, these data show that conditional ablation of c-Myc at the β-selection checkpoint did not affect the transition to the DN4 stage but resulted in the development of fewer DP cells (19% versus 82% adult, and 8% versus 48% embryonic DP cells, **Figure 5.6A**). This could either reflect reduced proliferation or increased apoptosis of cells traversing the preTCR checkpoint.

5.1.3 c-Myc ablation impairs preTCR dependent proliferation

Following assembly of the preTCR, developing thymocytes undergo rapid proliferation at the DN4 stage before progressing to the $CD4^{+}CD8^{+}$ DP stage. To address the role of c-Myc in this wave of proliferation, we compared the fraction of cycling cells in *Lck*Cre control and *Lck*Cre Myc$^{fl/fl}$ immature thymocytes. Thymocyte suspensions were surface stained to allow identification of the various thymocyte subsets, followed by intracellular staining with the DNA-intercalating dye 7-amino actinomycin D (7-AAD) and FACS analysis. Observation of 7-AAD staining in a linear mode allows discrimination of cycling cells in the S/G2/M phases of cell cycle from cells in the G1 phase, since DNA content doubles during normal cell cycle progression..

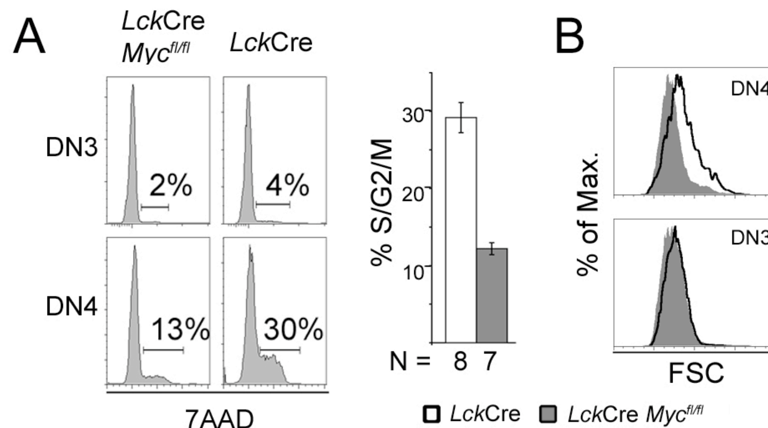


Figure 5.7: c-Myc ablation inhibits proliferation of DN4 stage thymocytes. (**A**) 7-AAD staining of permeabilized thymocytes. Thymocytes from the indicated mice were surface stained with lin antibodies as well as anti-CD44 and anti-CD25, followed by staining with 7-AAD and FACS analysis. Histograms depict 7-AAD staining of electronically gated $lin^{-}/CD44^{-}/CD25^{+}$ (DN3) or $lin^{-}/CD44^{-}/CD25^{-}$ (DN4) subsets. Percentages in histograms represent cells in S/G2/M phases of the cell cycle. The bar diagram represents cumulative measurements of 8 control and 7 *Lck*Cre-*Myc*$^{fl/fl}$ cycling DN4 cells. Error bars indicate standard deviation. (**B**) Forward Scatter (FSC) profiles of the indicated mice and subsets are shown to illustrate cell size.

At the resting DN3 stage, only 4% of *Lck*Cre and 2% of *Lck*Cre Myc$^{fl/fl}$ thymocytes were in the G2/S/M phases of the cell cycle. The fraction of *Lck*Cre control cells in the G2/S/M phases at the actively proliferating DN4 stage was 29.1±1.96%, while only 12.2±0.78% of the c-Myc deficient thymocytes were cycling (**Figure 5.7A**), suggesting that c-Myc was required for proliferation at the preTCR checkpoint. We also observed that *Lck*Cre Myc$^{fl/fl}$ DN4 stage cells were smaller (**Figure 5.7B**) than the equivalent *Lck*Cre cells, probably reflecting the contribution of c-Myc to cell growth.

5.1.4 c-Myc ablation does not negatively impact preTCR expression

To ensure that the ablation of c-Myc did not impact preTCR assembly we compared the expression of components of the preTCR complex in *Lck*Cre control and *Lck*Cre Myc$^{fl/fl}$ thymocytes. Semi-quantitative RT-PCR analysis using RNA prepared from sorted DN3 and DN4 stage thymocytes indicated that the expression of pTα (*Ptcra*) and TCRβ (*Tcrb*) was not impaired in *Lck*Cre Myc$^{fl/fl}$ mice. These cells expressed even higher levels of pTα mRNA compared to the equivalent *Lck*Cre control cells (**Figure 5.8A**). Intracellular TCRβ staining revealed that an equal fraction of DN4 stage thymocytes from *Lck*Cre Myc$^{fl/fl}$ and

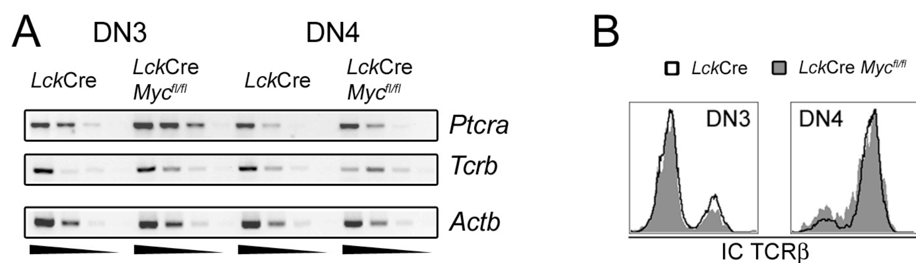


Figure 5.8: c-Myc ablation does not influence preTCR expression. (**A**) Semi-quantitative RT-PCR for pTα and TCRβ mRNA expression in DN3 and DN4 thymocytes. RT-PCR for β-actin (Actb) was used to control for equal loading. Similar results were observed in three independent experiments. (**B**) Intracellular TCRβ expression in DN3 and DN4 thymocytes. Cells were surface stained as in **res7A** followed by intracellular staining with anti-TCRβ antibodies and FACS analysis. Similar results were obtained in more than five independent experiments.

*Lck*Cre mice expressed TCRβ chains indicating that c-Myc deficiency did not negatively affect rearrangement or synthesis of TCRβ chains (**Figure 5.8B**).
These findings indicate that the defective thymic profiles of c-Myc deficient mice are not due to improperly assembled or otherwise dysfunctional preTCR, but do indeed result from impaired proliferation post β-selection.

5.1.5 c-Myc deficiency results in deregulation of cell cycle inhibitors

Although several genes involved in cell cycle progression and growth control were shown to be transcriptionally regulated by c-Myc (Dang, 1999; Zeller et al., 2003), the mechanism by which c-Myc mediates cell cycle progression remains

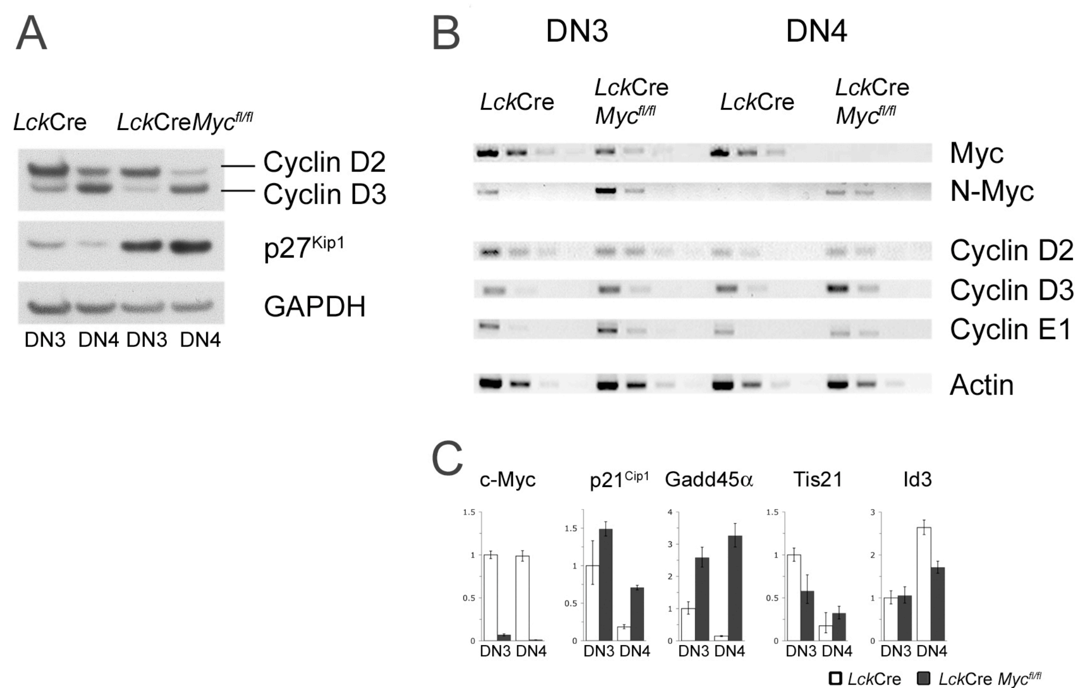


Figure 5.9: Elevated levels of cell cycle inhibitors in *Lck*Cre Myc$^{fl/fl}$ thymocytes. (**A**) Western Blot of sorted cells (2x 10^6 per lane) probed with antibodies detecting the indicated proteins. Data are representative of 3 independent experiments. (**B**) Semi-quantitative RT-PCR with 5-fold serial dilutions for cell cycle related genes performed on cDNA obtained from FACS sorted DN3 and DN4 thymocytes. (**C**) Quantitative RT-PCR analysis using cDNA prepared from similarly sorted cells was performed in triplicate for the indicated genes. White bars represent results for *Lck*Cre control dark grey bars represent results for *Lck*Cre *Myc*$^{fl/fl}$ mice. Y-axis indicates fold difference in relative expression levels normalized to the relative expression level

unclear, especially in thymocytes. To trace the impact of c-Myc ablation on genes involved in proliferation we compared their expression in *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre DN3 and DN4 stage thymocytes by semiquantitative and quantitative PCR as well as Western blots (**Figure 5.9**). These analyses revealed strikingly elevated protein levels of the cell cycle inhibitor p27^{Kip} in DN3 and DN4 thymocytes of *Lck*Cre Myc$^{fl/fl}$ mice (**Figure5.9A**). c-Myc deficient DN4 stage cells also showed elevated expression of the growth arrest and DNA-damage-inducible factor 45 alpha (Gadd45α) and to a lesser extent of the cell cycle inhibitor p21^{Cip1} (**Figure 5.9C**). Both message and protein levels of the growth promoting Cyclins D2 and D3 appeared unchanged, and the mRNA levels of Cyclin E1 were only modestly increased (**Figure 5.9A and B**). This is noteworthy considering that Cyclin D3 (**Figure 5.9A**), has previously been reported to control the proliferative expansion of late stage DN and ISP thymocytes (Sicinska et al., 2003). Thus, it is likely that the proliferation defect following c-Myc ablation is related to the elevated expression of cell cycle inhibitors such as p27^{Kip}, p21^{Cip1}, and Gadd45α, which are normally negatively controlled by c-Myc (Cairo et al., 2005). This notion was further supported by the unchanged expression of genes previously implicated in thymocyte proliferation. These included the inhibitory protein Tis21, described to regulate stage specific proliferation in fetal thymocytes (Konrad and Zuniga-Pflucker, 2005) and the Id3 inhibitor of E2A activity (Engel and Murre, 2004) previously shown to be induced after preTCR signaling (Bain et al., 2001). Id 3 was modestly elevated at the DN4 stage approximately at 70% of control levels (**Figure 5.9C**). c-Myc ablation was linked to a transcriptional upregulation of N-Myc at the DN3 and DN4 stages that was insufficient to compensate for the c-Myc mediated defect.

These observations indicate that c-Myc impacts proliferation at the preTCR checkpoint by affecting the expression of cell cycle inhibitors, especially p27^{Kip} and Gadd45α rather than directly affecting the expression of the cell cycle promoting cyclins or other genes reported to promote thymocyte expansion downstream of of the preTCR.

5.1.6 c-Myc$^{-/-}$ DN3 cells have the potential to differentiate

As shown above, *Lck*Cre Myc$^{fl/fl}$ thymocytes developed to the CD4^{+}CD8^{+} DP stage despite reduced proliferation at the DN4 stage, indicating that c-Myc ablation did not influence their differentiation potential. However, we could not completely rule out the possibility that DP cells resulted from cells that deleted their Myc genes particularly late, e.g. only at the DN4 stage. To precisely address the differentiation potential of c-Myc deficient DN3 cells we therefore crossed *Lck*Cre Myc$^{fl/fl}$ mice onto the *Rag2*$^{-/-}$ background. These mice showed a complete block at the DN3 stage of thymocyte development allowing for a more homogeneous pool of cells to study. We induced preTCR signaling in *Lck*Cre

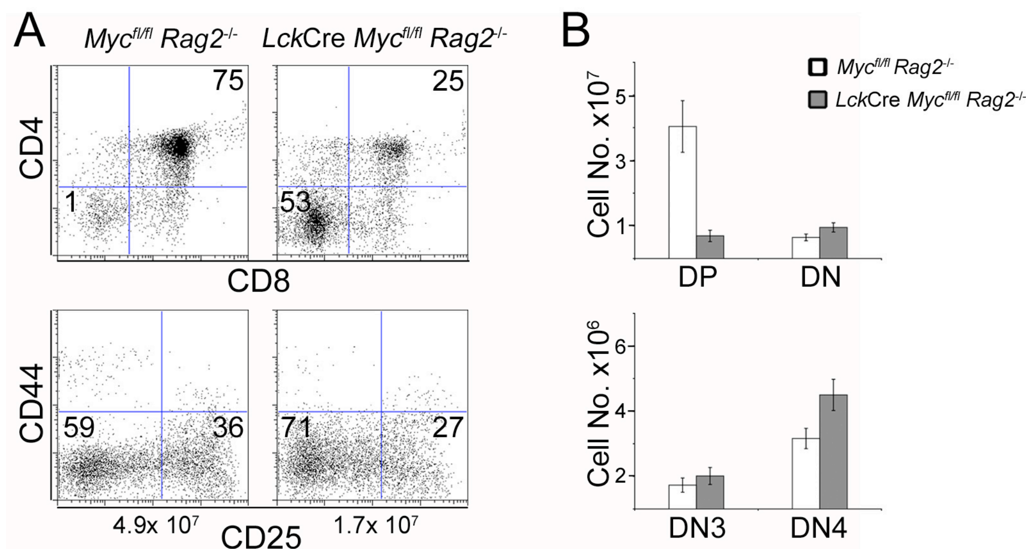


Figure 5.10: preTCR like signals induce differentiation of *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ thymocytes. (**A**) FACS profiles for CD4/CD8 (upper panels) and lin^{-}/CD44/CD25 expression 4 days after injection (i. p.) of 50 µg α-CD3ε mAb. Numbers given represent the percentages of cells in the respective quadrants. (**B**) Cellularity was calculated from total thymocyte numbers and the fraction of cells in the indicated subsets. Error bars indicate standard deviation. 11 *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ and 6 *Lck*Cre *Rag2*$^{-/-}$ were analyzed to obtain statistics.

Myc$^{fl/fl}$ *Rag2*$^{-/-}$ and control *Lck*Cre *Rag2*$^{-/-}$ mice by injecting α-CD3ε mAb (50 μg/ mouse) and analyzed thymic development four days later with respect to cellularity and thymocyte subset distribution. This approach allowed for orchestrated onset of preTCR signaling, since all DN3 cells received the signal at the same time.

Developmental progression was examined by staining for surface expression of CD4, CD8, CD25, and CD44 (**Figure 5.10A**). Both *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$ and *Lck*Cre *Rag2*$^{-/-}$ mice progressed developmentally in response to the treatment, however *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$ thymi contained 3-5 fold fewer thymocytes than *Lck*Cre *Rag2*$^{-/-}$ controls. The two mouse strains had comparable numbers of DN3 and DN4 stage thymocytes indicating that c-Myc ablation did not inhibit progression to the DN4 stage. The reduced cellularity of *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ thymi compared to *Lck*Cre *Rag2*$^{-/-}$ controls was entirely reflected in the reduced fraction (25% versus 75%) and number ($4.0x10^7 \pm 7.9x10^6$ versus $6.9x10^6 \pm 1.73x10^6$, **Figure 5.10B**) of DP cells. These data suggested that c-Myc was not required for the preTCR mediated developmental transition from the DN3 to the DP stage.

To rule out the possibility that the progressing cells in α-CD3ε treated *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ mice may have "escaped" timely Cre-mediated deletion of *Myc*, we performed semi-quantitative PCR analyses using genomic DNA isolated from sorted DN3, DN4 and DP cells. The floxed *Myc* allele was barely detectable in DN4 and DP stage *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$ thymocytes indicating that these cells

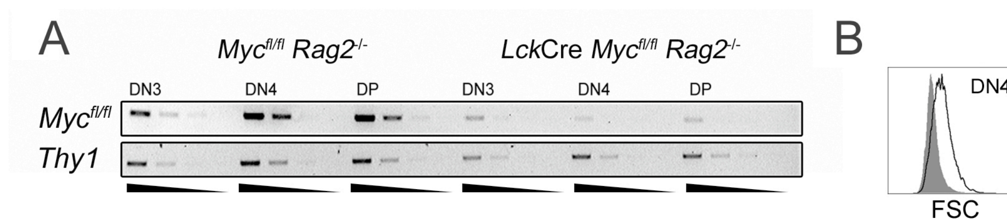


Figure 5.11: Efficient deletion of *Myc* in *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$ mice. (**A**) Semi-quantitative PCR with 5-fold serial dilutions was performed to detect the floxed *Myc* allele. Genomic DNA was obtained from FACS sorted DN3, DN4 and DP cells. (**B**) FSC as observed by FACS to assess cell size of DN4 stage thymocytes.
Solid grey: *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$, white: *Lck*Cre Rag2$^{-/-}$

had undergone efficient *Myc* deletion (**Figure 5.11A**). Moreover, *Lck*Cre Myc$^{fl/fl}$ Rag2$^{-/-}$ DN4 thymocytes were smaller than *Lck*Cre Rag2$^{-/-}$ cells (**Figure 5.11B**), likewise indicating efficient c-Myc ablation before the DN4 stage. Thus, *Lck*Cre Myc$^{fl/f}$ Rag2$^{-/-}$ DN3 thymocytes developed to the DP stage despite the lack of c-Myc.

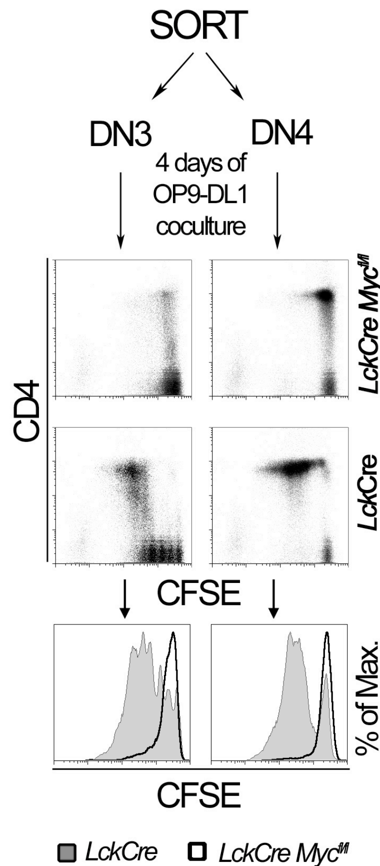


Figure 5.12: c-Myc deficient thymocytes differentiate without proliferation. FACS sorted DN3 and DN4 cells from the indicated mice were labeled with CFSE and cocultured on OP9-DL1 cells for 4 days. They were then stained with antibodies against CD4, CD8 or TCRαβ and analyzed by flow cytometry. Upper panels depict two-parameter dot plots of CD4 versus CFSE staining of the indicated subsets and mice. Lower panels show histogram overlays comparing CFSE in *LckCre* versus *LckCre Myc*$^{fl/fl}$ cells after the coculture.

5.1.7 c-Myc deficient thymocytes differentiate without cell division

5.1.7.1 DP transition does not require cell division

To further determine whether cell division was required for developmental progression, we cocultured immature *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre control thymocytes with OP9-DL1 (Schmitt and Zuniga-Pflucker, 2002) cells. Sorted DN3 and DN4 stage thymocytes from *Lck*Cre control and *Lck*Cre Myc$^{fl/fl}$ mice were labeled with 5,6-carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) and cocultured on OP9-DL1 cells for four days before staining for CD4 and CD8 surface expression and FACS analysis. A substantial fraction of c-Myc deficient DN3 stage thymocytes upregulated CD4 (**Figure 5.12**) and CD8 (**Figure

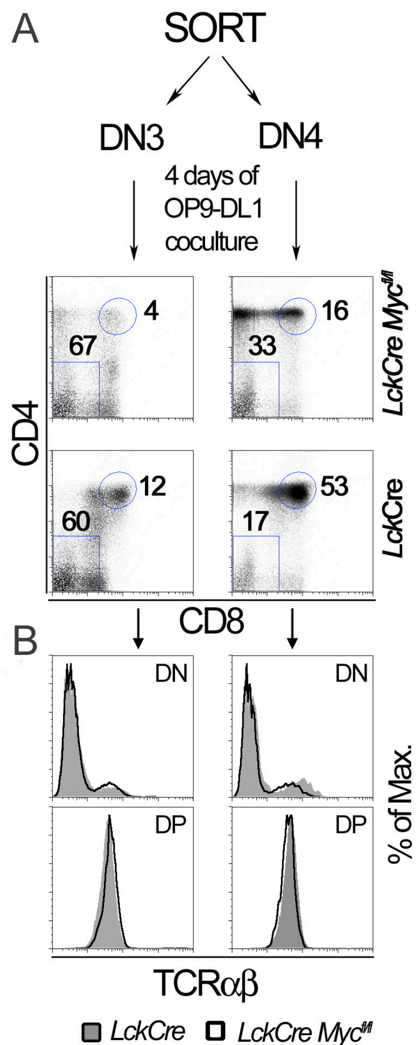


Figure 5.13: Normal TCRαβ levels on c-Myc deficient DP cells. The experimental setup is the same as in Figure 5.12. (**A**) (Upper) Two-parameter dot plots of CD4 versus CD8 surface staining of the indicated cells and mice. Numbers represent the percentage of total events in the shown gates. (**C**) TCRαβ surface expression in the gates shown in B. *In vitro* differentiation was observed in three independent experiments.

5.13A) surface expression without any cell division while the developing fraction of *Lck*Cre control DN3 cells had undergone four to five cell divisions (**Figure 5.12**, upper panels). Likewise, c-Myc deficient DN4 cells did not divide but upregulated CD4 and CD8 surface expression.

5.1.7.2 c-Myc deficient DP cells express TCRαβ

To determine whether these DP cells had undergone proper differentiation we examined their surface expression of TCRαβ (**Figure 5.13B**). *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre control DP cells developing in OP9-DL1 cocultures expressed comparable levels of TCRαβ on their surface.

These findings provided both *in vivo* and *in vitro* evidence that neither c-Myc signaling nor proliferation was required for developmental progression past the preTCR checkpoint.

5.1.8 c-Myc ablation does not compromise survival of developing thymocytes

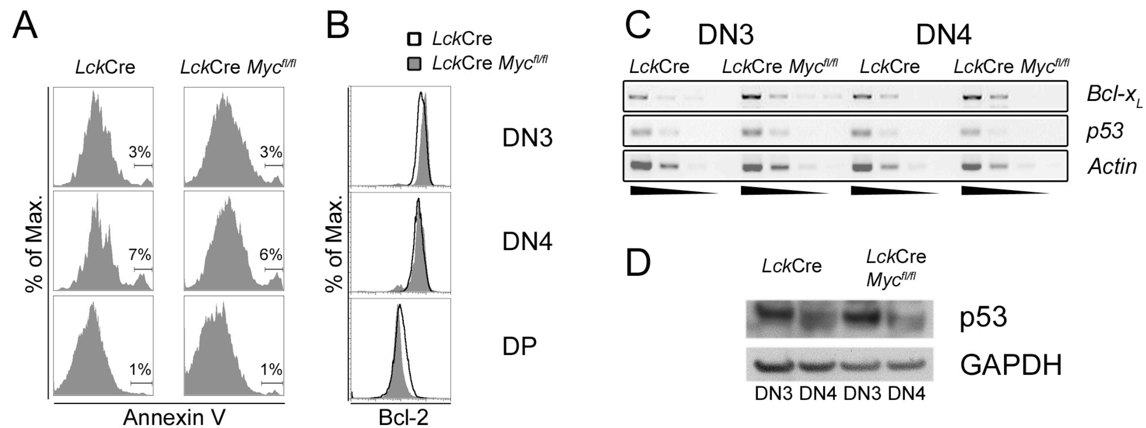


Figure 5.14: c-Myc ablation does not affect survival of developing thymocytes. (**A**) Annexin V staining of primary thymocytes. Thymocytes from the indicated mice were stained with antibodies against CD4 and CD8 or against lin, CD44 and CD25 followed by Annexin V and FACS analysis. Histograms of Annexin V staining are electronically gated on the indicated subsets. (**B**) The same subsets were also analyzed with respect to the expression of intracellular Bcl-2 levels. (**C**) Expression levels of Bcl2l1 (Bcl-x_L) and Trp53 (p53) mRNA in FACS sorted DN3 and DN4 cells as determined by semi-quantitative RT-PCR with 5-fold serial dilutions. Semiquantitative RT-PCR for β-actin was used as quantity control (Actin). Similar results were obtained in three independent experiments. (**D**) Protein levels of p53 in FACS sorted cells (2x 10^6 per lane) as determined by Western blot.

To examine whether c-Myc deficiency had an effect on the survival of developing thymocytes, we compared *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre control thymocytes with respect to their fraction of apoptotic cells determined by Annexin V$^+$ staining. Likewise, the expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and the pro-apoptotic protein p53 were examined. To this end we stained primary thymocytes with Annexin V as well as antibodies directed against surface markers that allow electronic gating of specific thymocyte subsets (**Figure 5.14A**). *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre control mice had comparable fractions of Annexin V$^+$ cells in the DN3, DN4, and DP subsets, indicating that the c-Myc deficiency did not affect the survival of developing thymocytes. Intracellular

staining with antibodies against Bcl-2 revealed that *Lck*Cre Myc$^{fl/fl}$ and *Lck*Cre thymocytes expressed comparable levels of Bcl-2 at the DN3, DN4, and DP stages further supporting this notion (**Figure 5.14B**). We also analyzed the expression levels of Bcl-x_L (*Bcl2l1*) and p53 (*Trp53*) mRNA using semi-quantitative RT-PCR (**Figure 5.14C**) and of p53 protein using Western blot (**Figure 5.14D**). The expression level of p53 was unchanged, while Bcl-x_L expression was modestly elevated in the absence of c-Myc.

In summary, these data indicate that c-Myc ablation at the DN3 stage did not impair the preTCR dependent survival signals and that the reduced thymic cellularity was entirely the result of reduced proliferation.

Taken together the presented data show that induction of preTCR signaling resulted in rapid elevation of c-Myc protein levels. Cre mediated thymocyte specific ablation of c-Myc in CD25$^+$CD44$^-$ DN3 thymocytes reduced proliferation and cell growth at the preTCR checkpoint, resulting in thymic hypocellularity and a severe reduction in CD4$^+$CD8$^+$ DP thymocytes. In contrast, c-Myc deficiency did not inhibit preTCR mediated differentiation or survival. Myc$^{-/-}$ DN3 cells progressed to the DP stage and upregulated TCR$\alpha\beta$ surface expression in the absence of cell proliferation, *in vivo* as well as *in vitro*. These observations indicate that distinct signals downstream of of the preTCR are responsible for proliferation *versus* differentiation, and demonstrate that c-Myc is only required for preTCR induced proliferation but is dispensable for developmental progression from the DN to the DP stage

5.2 Survival signals at the β-selction checkpoint- a role for Akt?

5.2.1 Akt activation in response to preTCR signaling

Data presented so far indicate that distinct signals downstream of the preTCR are responsible for proliferation *versus* differentiation and survival. The serine/threonine kinase Akt has been described as a mediator of survival signals (Nicholson and Anderson, 2002). Recently, Akt has been implicated as a downstream mediator of Notch induced survival at the preTCR checkpoint in DN3 cells derived from fetal liver cells *ex vivo* (Ciofani and Zuniga-Pflucker, 2005). Thus, we next addressed the role of Akt at the β-selection checkpoint in adult thymocytes.

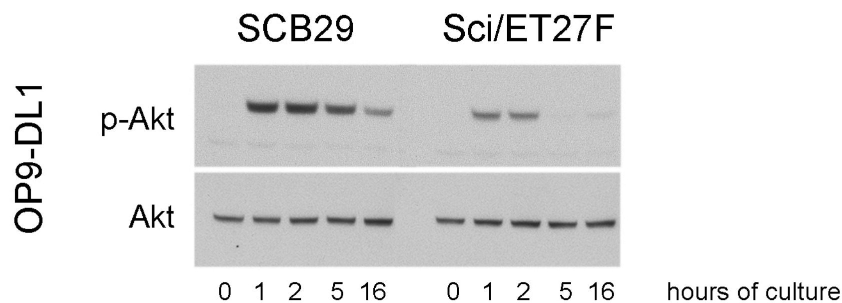


Figure 5.15: preTCR enhances Akt activation. Sorted SCB.29 and SciET27F that had been cocultured on OP9-DL1 cells for the indicated amount of time were subjected to Western blot analysis for p-Akt (Thr308) and total Akt. An amount corresponding to 2x 10^6 cells per lane was loaded.

To this end we employed the preTCR$^-$ SciET27F and the preTCR$^+$ SCB.29 cell lines. ScieET27F was derived from a spontaneously occurring thymoma in Scid mice while SCB.29 cells were obtained from SciET27F by transfection with a TCRβ construct (Groettrup et al., 1992). Cell lines were cocultured on OP9-DL1 and OP9-GFP stromal cells for 0, 1, 2, 5, and 16 hours. Live cells were sorted and subjected to Western blot analysis for phosphorylated Akt (p-Akt) (**Figure**

5.15). Upon change of culture conditions, i.e. after placement on feeder cells, Akt phosphorylation at threonine 308 (resulting in activation of the kinase) was strongly induced. While Akt activation could be observed both in SciET27F as well as SCB.29 cells, this effect was considerably stronger in the presence of preTCR, i.e. in SCB.29 cells. This observation indicated that preTCR signals enhanced Akt activation and led to sustained p-Akt levels, suggesting that Akt might be involved in preTCR downstream of signaling.

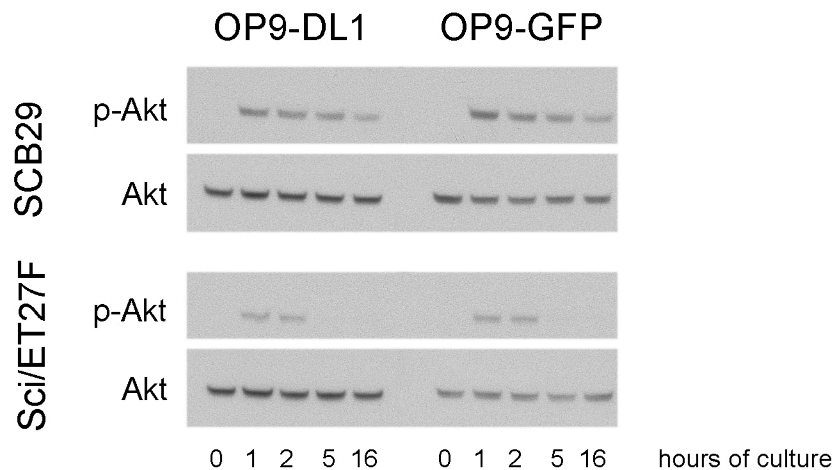


Figure 5.16: Akt activation by preTCR is independent of Notch. Sorted SCB.29 and SciET27F that had been cocultured on OP9-DL1 and OP9-GFP cells for the indicated amount of time were subjected to Western blot analysis for p-Akt (Thr308) and total Akt. An amount corresponding to 2x 10^6 cells per lane was loaded. These samples were identical to those in **Figure resX** but were run on multiple gels to allow different comparisons.

In addition, we observed that this phenomenon was completely independent of Notch signals provided by the stroma cells, since p-Akt levels were identical in cells cultured on OP9-DL1 as compared to OP9-GFP (**Figure 5.16**).

These data suggest, that Akt activation in adult cells might not depend on Notch but rather be a result of preTCR signaling and other pathways resulting in Akt mediated survival.

5.2.2 Akt1 does not overcome the need for Notch signals at the β-selection checkpoint

To further validate our observation that Akt activation downstream of the preTCR was independent of Notch signaling in adult thymocytes, we sought to analyze a mouse model for constitutively active Akt in the thymus. To this end we employed MyrAkt mice (Malstrom et al., 2001). These mice have been generated by Tsichlis and colleagues and express a transgene encoding a constitutively active form of Akt under the proximal *Lck* promoter. Therefore, they express activated Akt in the thymus starting at the DN2 to DN3 transition.

DN3 cells form MyrAkt mice were sorted and cocultured on OP9-DL1 and OP9-GFP stroma cells to examine the need for Notch signaling in the presence of activated Akt at the preTCR checkpoint. Different conditions regarding the availability of Notch signaling were analyzed. Namely, cocultured on OP9-DL1 (Notch signals), OP9-DL1 with 1 μM γ-secretase inhibitor (GSI, no Notch signal

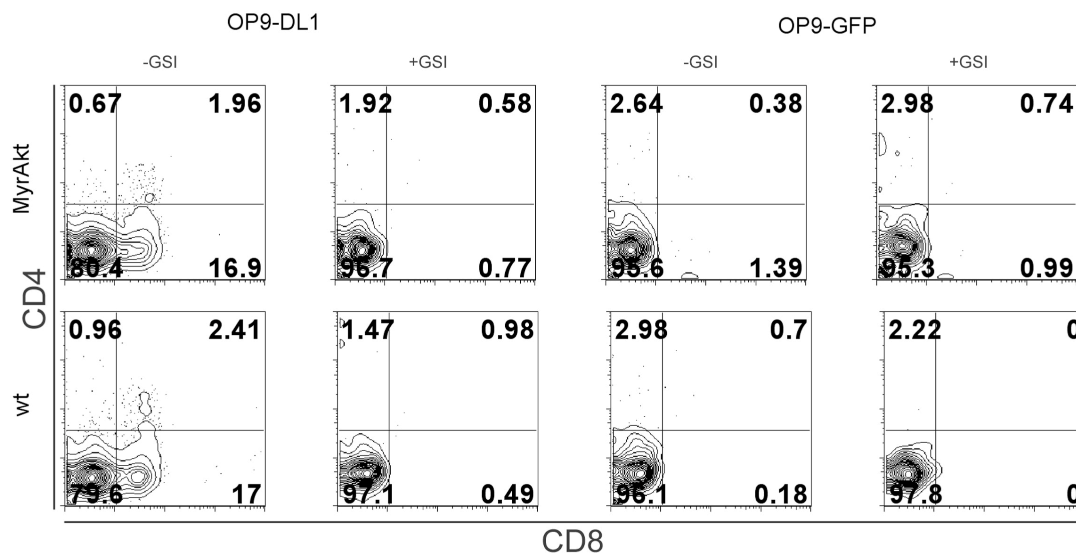


Figure 5.17: Constitutive activation of Akt does not overcome the need for Notch signaling. Sorted MyrAkt and wt DN3 cells were cocultured on OP9-DL1 and OP9-GFP cells for seven days in the presence or absence of 1 μM GSI. Histograms depict FACS analysis for CD4/CD8 surface expression on these thymocytes.

transduction inside thymocytes), OP9-GFP (no DL1 expressed, therefore no Notch signaling triggered from stromal cells), and OP9-GFP with 1µM GSI (no DL1 ligand expressed on the stromal cells and no signal transduction in thymocytes). Thus, three conditions lacking Notch signaling were compared with the situation in the presence of Notch signals. Seven days after sorting cells were stained for surface expression of CD4 and CD8 and were subjected to FACS analysis (**Figure 5.17**). Interestingly, in the absence of Notch signaling there was no developmental progression of MyrAkt DN3 cells. Cell numbers were unchanged as well indicating that the activation of Akt did not result in improved survival (data not shown).

Therefore, activation of Akt in thymocytes was not sufficient to overcome the need for Notch signals, and MyrAkt DN3 cells were phenotypically identical to wt control cells.

5.3 The role of Wnt/ β-catenin signaling at the preTCR checkpoint

Wnt/ β-catenin signaling has been implicated in the development of multiple organ systems. Previous observations indicate that Wnt signaling is important for HSC interaction with the bone marrow stem cell niche. Developing thymocytes interact with the microenvironment provided by a multitude of thymic stromal cells, as well. Several interactions have been described that are vitally important for developmental progression within the thymus. In addition, it has been reported that β-catenin stabilization can circumvent β-selection in mice (Gounari et al., 2001). Notch signaling, another signaling cue provided by stroma cells, is indispensable for progression past the preTCR checkpoint in wt cells. We therefore sought to address the roles of Notch and Wnt signaling and their potential interplay at the preTCR checkpoint in greater detail. To this end OP9-DL1 and OP9-GFP cocultures were employed, representing presence and absence of Notch signals respectively.

5.3.1 Background: β-catenin stabilization in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice

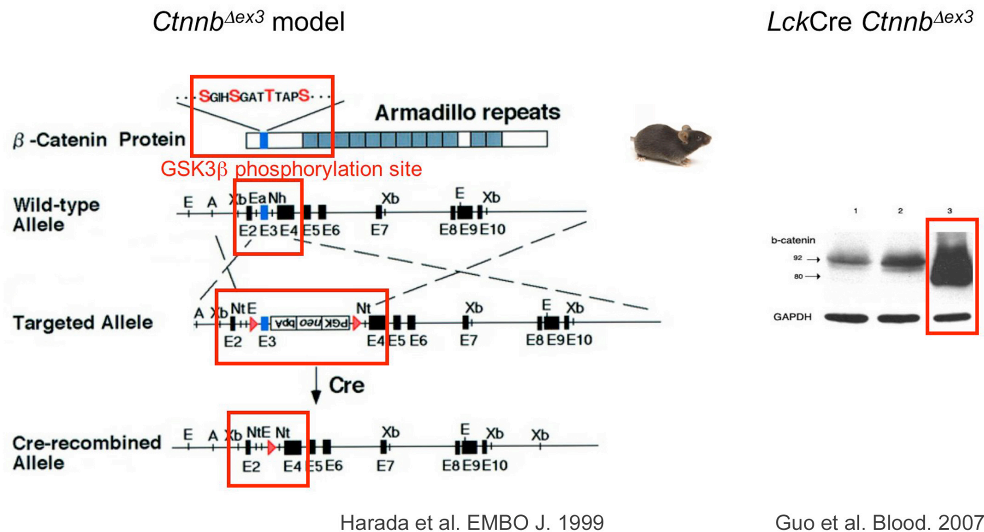


Figure 5.18: *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice. The schematic representation illustrates the genetic modifications made to the *Cttnb* locus. A Western blot is shown on the right. Single cells suspensions were obtained from thymi of two wt mice (lanes 1 and 2) and one *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mouse (lane 3). Membranes were probed with antibodies detecting β-catenin. *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice express high levels of a shorter form of β-catenin.

The β-catenin stabilization model used here was generated by Taketo and colleagues (Harada et al., 1999). As depicted in **Figure 5.18** exon 3 of the *Ctnnb* gene was flanked by LoxP sites resulting in excision of this entire exon upon Cre mediated recombination. Exon 3 encodes four critical GSK3β phosphorylation sites that, if phosphorylated, lead to ubiquitination and subsequent degradation of β-catenin. Ablation of this sequence results in a degradation resistant, stable form of β-catenin that translocates to the nucleus to initiate Tcf/ Lef mediated transcription. Crossing of *Ctnnb*$^{\Delta ex3}$ with *Lck*Cre transgenic mice led to the *Lck*Cre *Ctnnb*$^{\Delta ex3}$ model. Catenin stabilization started at the DN3 stage (**Figure 5.19**) and was complete at the DP stage with most cells expressing elevated β-catenin levels compared to controls.

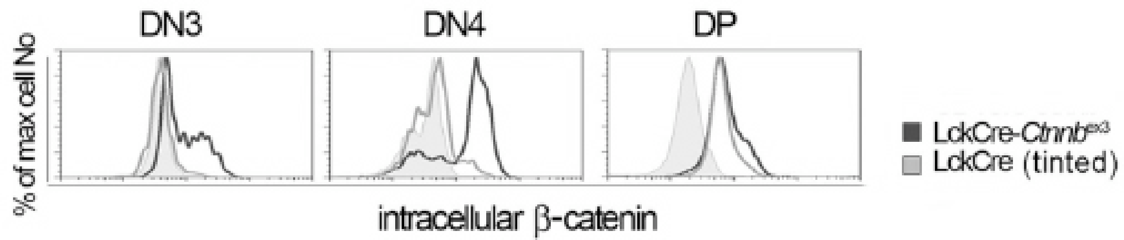


Figure 5.19: Course of β-catenin stabilization during *Lck*Cre *Ctnnb*$^{\Delta ex3}$thymocyte development. Thymocytes from the indicated mice were surface stained for expression of CD4 and CD8, or with a cocktail of lineage specific antibodies combined with staining for surface expression of CD44 and CD25. Cells were then permeabilized and stained with anti-β-catenin. Histogram overlays are representative of four independent experiments. (The figure also shows, in light grey and not tinted, β-catenin stabilization in *CD4*Cre *Ctnnb*$^{\Delta Ex3}$ mice that are not discussed here).

Thus, *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice represent a model system to study the effects of conditional stabilization of β-catenin on development past the preTCR checkpoint. Data presented in the following were obtained from FACS sorted cells cultured ex vivo on OP9 cocultures as indicated.

5.3.2 Conditional β-catenin stabilization overrides the need for Notch signals at the DN3 stage

As mentioned before, stabilization of β-catenin in the thymus has previously been shown to promote development to the DP stage without preTCR signaling (Gounari et al., 2001) indicating that it must convey a strong differentiation signal to thymocytes overcoming a checkpoint that is normally highly restrictive. Another absolute requirement for developmental transition of DN3 thymocytes to the DP stage is Notch signaling (Maillard et al., 2006). Therefore, it was of interest to establish whether β-catenin stabilization could also overcome the need for Notch signals.

To address this question, DN3 stage thymocytes were sorted from *Lck*Cre *Ctnnb*$^{\Delta ex3}$ as well as control mice. Cells were plated on OP9 stromal cell cultures. Different conditions regarding the availability of Notch signaling were analyzed. Namely, DN3 cells were plated on OP9-DL1 (Notch signals), OP9-DL1 with 1 µM γ-secretase inhibitor (a concentration previously established to block all Notch activity (Garbe et al., 2006)) (GSI, no Notch signal transduction inside thymocytes), OP9-GFP (no DL1 expressed, therefore no DL1 mediated Notch signaling triggered from stromal cells), and OP9-GFP with 1µM GSI (no DL1 ligand expressed on the stromal cells and no signal transduction in thymocytes). Thus, three conditions lacking Notch signaling were compared with the situation in the presence of Notch signals.

Sorted thymocytes were cocultured for three days and analyzed by FACS for surface expression of CD4 and CD8. Wilde-type cells developed only in the presence of Notch signals, i.e. on OP9-DL1 cells without GSI (**Figure 5.20**). On the day of analysis approximately 5% of thymocytes were DP whereas about 78% of cells remained DN (**Figure 5.20**, lower panels). In the absence of Notch signals no differentiation could be observed and close to 100% of cells were found within the DN compartment.

Interestingly, this differed significantly in cocultures seeded with *Lck*Cre *Ctnnb*$^{\Delta ex3}$ DN3 thymocytes. Three days after sorting these cells had developed to the DP

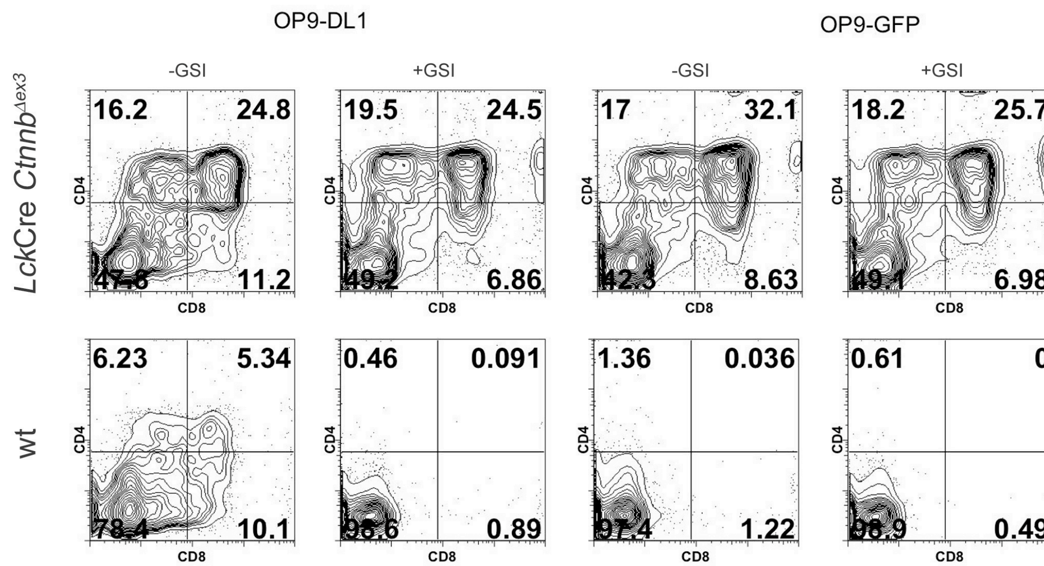


Figure 5.20: Notch is dispensable for development of *Lck*Cre *Ctnnb1*$^{\Delta ex3}$ DN3 cells. Sorted DN3 cells were cocultured on OP9-DL1 and OP9-GFP cells for 3 days with γ-secretase inhibitor (GSI) or DMSO. Cells were harvested and stained for surface markers CD4, CD8, and TCRβ. Numbers indicate the percentages of cells in the quadrants shown.

stage (between 25% and 32%), with a significant percentage (between 16% and 18%) of cells expressing only CD4. There was no difference in the percentages of DP cells obtained on OP9-DL1 cocultures in the presence or absence of GSI, or on OP9-GFP plus GSI (25% DP cells under either condition). On OP9-GFP without GSI 32% of DP cells were obtained.

Therefore, Notch signaling was dispensable for *ex vivo* developmental progression of cells that express a stabilized form of β-catenin.

5.3.3 Reduced numbers of TCRβ^{+} cells upon β-catenin stabilization *ex vivo*

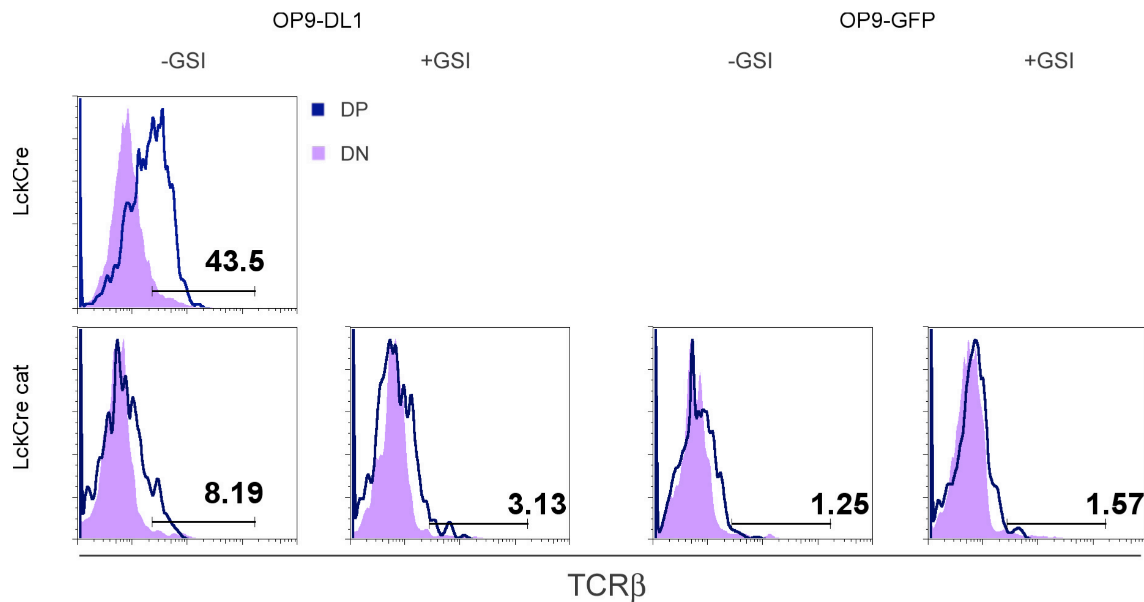


Figure 5.21: *Ex vivo* differentiation of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ DN3 cells without preTCR. Thymocytes differentiated on *ex vivo* OP9 cocultures for three days with the indicated treatments. Cells were stained with antibodies against CD4, CD8, and TCRβ and analyzed by FACS. Data were electronically gated to produce histogram overlays for TCRβ expression levels in the indicated subsets.

Given the fact that most DP cells developing in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice do not express TCRβ (Gounari et al., 2001; Guo et al., 2007) these data would indicate that β-catenin stabilization can overcome the need for preTCR as well as Notch. In order to determine the presence of TCRαβ on DP *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells that had been differentiated from DN3 cells *ex vivo*, we analyzed TCRβ surface expression by FACS (**Figure 5.21**). While TCRβ expression was not detectable on DN cells, almost half of wt DP cells (43.5%) expressed it. This was different in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ derived DP cells, where only 8% of cells diplayed TCRβ surface expression in the presence of Notch that was even further reduced to between 1.6% and 3.1% if Notch signaling was absent. These data indicate that β-catenin stabilization enables cells to develop without preTCR and Notch *ex vivo*. preTCR

cannot be directly detected by FACS, and this approach therefore assumes that preTCR signaling results in measurable TCRαβ surface expression. It cannot be ruled out that observed defects in TCRαβ expression are due to impaired TCRα rearrangement, while preTCR levels are not perturbed (but cannot be detected).

5.3.4 No Notch requirement in the complete absence of preTCR in cells with β-catenin stabilization

Data presented so far indicate that indeed Notch signals are not required in the absence of preTCR if cells express stabilized β-catenin. To exclude the possibility that preTCR is required at low levels that are too low to result in TCRαβ surface expression or that preTCR is required in general while low TCRαβ surface expression is a consequence of impaired TCRα rearrangement, we crossed *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice onto the *Rag2*$^{-/-}$ background to obtain *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ mice. As mentioned before, *Rag*$^{-/-}$ mice cannot recombine the TCR genes and therefore lack TCRβ expression resulting in preTCR deficiency. DP cells that have been shown to develop in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ mice are therefore preTCR deficient (Gounari et al., 2001) but whether this was Notch dependent is unknown.

To address this question we sorted DN3 cells from *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice, and cocultured them on OP9-DL1 and OP9-GFP stromal cells in the presence and absence of 1 µM GSI as above. Three, four, six, and seven days later cells were stained for CD4 and CD8 surface expression and subjected to FACS analysis. Similar to our observations in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells, *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ DN3 cells were able to differentiate in the absence of Notch signaling (between 22- 31% DP cell, **Figure 5.22**). This is depicted here for Day 6 of observation but was true at all time points (data not shown).

Thus, we established that even in the complete absence of preTCR signals β-catenin stabilization enables developing thymocytes to differentiate in the absence of Notch ligands *ex vivo*. This indicates that β-catenin might integrate several maturation signals in the DN to DP transition of thymocytes.

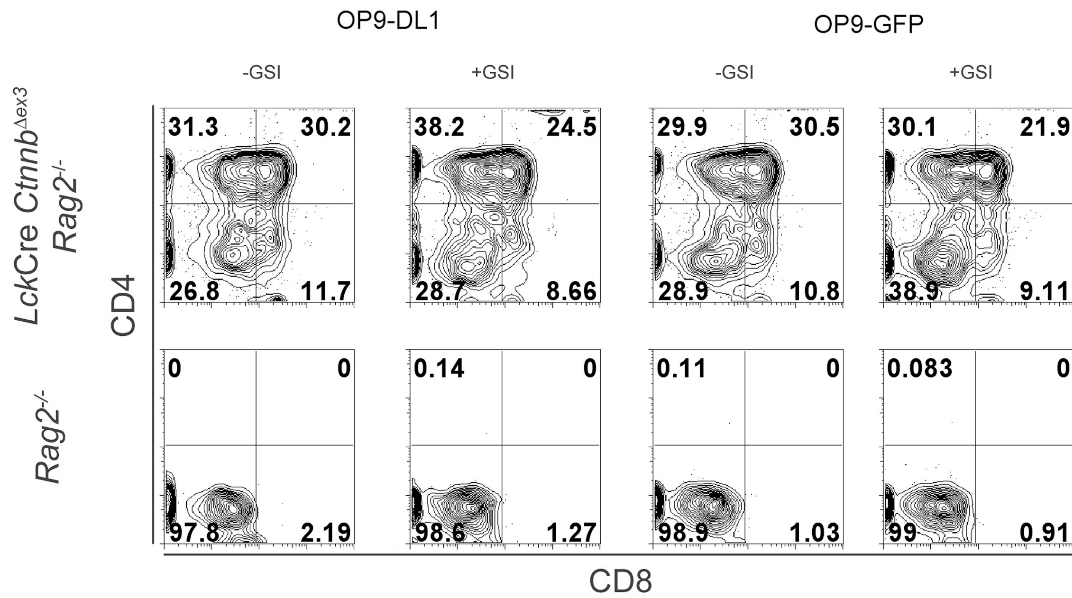


Figure 5.22: Development without preTCR and Notch in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ cells. *Lck*Cre *Ctnnb1*$^{\Delta ex3}$ *Rag2*$^{-/-}$ DN3 cells develop efficiently with and without Notch signaling on OP9 cocultures in the absence of any preTCR. FACS analysis of sorted DN3 cells after four days of coculture on the indicated stromal cells in the absence and presence of 1 μM GSI.

5.3.5 β-catenin stabilization and preTCR deficiency accelerate differentiation

When comparing developmental progression of *Lck*Cre, *Lck*Cre *Ctnnb*$^{\Delta ex3}$, and *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ DN3 cells *ex vivo*, striking differences became apparent. In **Figure 5.23** CD4/ CD8 surface profiles of cells cocultured on OP9-DL1 and OP9-GFP stromal cells for three days are depicted. While approximately 4% of control cells were DP and 88% DN after three days of coculture on OP9-DL1 one (and none in the absence of Notch signals), only 67% of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells remained DN, with 11% DP on both OP9-DL1 and OP9-GFP cocultures. This trend was further enhanced in the complete absence of preTCR with 23% DP and only 39% DN *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ on OP9-DL1 (22% and 46% respectively on OP9-GFP) cocultures. In the absence of Notch

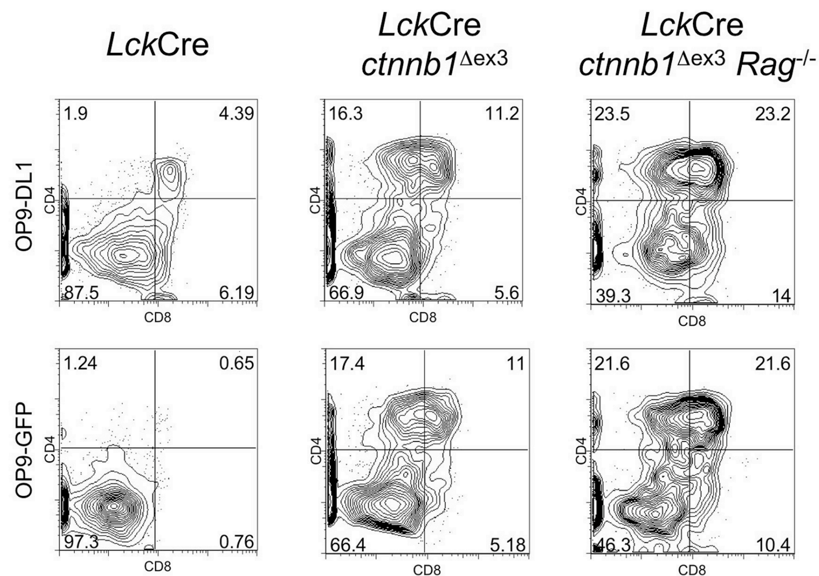


Figure 5.23: β-catenin stabilization accelerates differentation *ex vivo*. CD4/ CD8 surface profiles were obtained by FACS analysis after three days of coculture on the indicated stromal cells.

signals cells lacking β-selection progress faster (22% of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ cells versus 11% of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells). Similar results were obtained after 4, 6, and 7 days of coculture (data not shown).

These data indicate that β-catenin stabilization accelerates differentiation. This is especially profound in the absence of β-selection, i.e. in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ cells. Recent evidence supports the notion that DN3 cells that have undergone β-selection (DN3b) are no longer dependent on Notch signals for differentiation (Taghon et al., 2006). In this context our data indicate that Wnt/ β-catenin signaling might be a signaling cue normally obtained after β-selection that drives differentiation of such DN3b cells.

5.3.6 Proliferation in the absence of Notch in developing *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells

The presented evidence indicates that proliferation and differentiation may result from independently mediated signals downstream of the preTCR. Given the observed differentiation in the absence of Notch of DN3 stage thymocytes expressing stabilized β-catenin, it was further of interest to establish the contribution of Notch to proliferation in such cells.

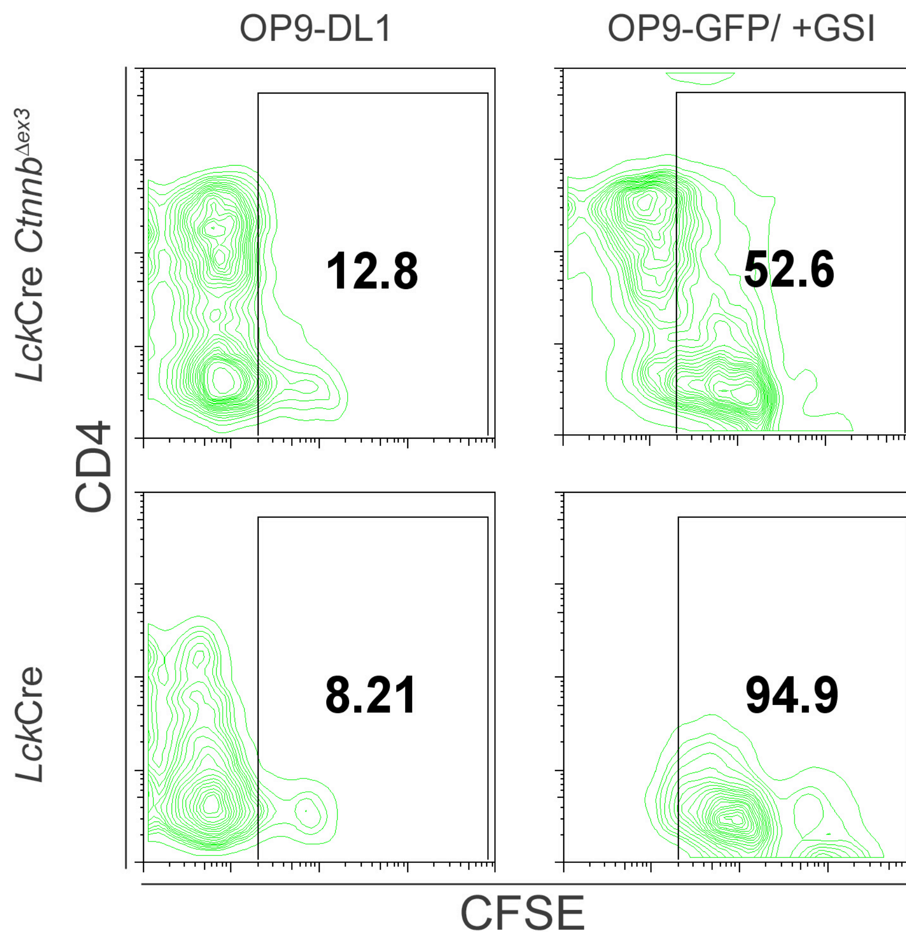


Figure 5.24: Proliferation in the absence of Notch signaling in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells. DN3 stage thymocytes were sorted from the indicated mice and labeled with CFSE. Cells were cocultured on the indicated stromal cells for six days and stained for CD4 and CD8 surface expression. Histograms depict CD4 versus CFSE staining. Higher levels of CFSE indicate that cells had divided to a lesser extent.

FACS sorted DN3 cells from *Lck*Cre *Ctnnb*$^{\Delta ex3}$ and *Lck*Cre mice were labeled with CFSE to monitor cell division. Thymocytes were then cocultured on OP9-DL1 and OP9-GFP stromal cells in the presence or absence of 1 µM GSI for six days and stained for surface expression of CD4 and CD8. Histograms presented in **Figure 5.24** depict CD4 expression versus CFSE staining for cells cultured on OP9-DL1 (presence of Notch signals) and OP9-GFP plus 1 µM GSI (complete absence of Notch signals). Similar results as on OP9-GFP plus 1 µM GSI were observed on OP9-DL1 plus 1 µM GSI and OP9-GFP alone (data not shown).

In the presence of Notch almost all *Lck*Cre *Ctnnb*$^{\Delta ex3}$ and *Lck*Cre cells had divided multiple times as reflected in the progressive dilution of the CFSE signal intensity (**Figure 5.24**). More specifically, all *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells that had differentiated and diplayed upregulated levels of CD4 and/ or CD8 surface expression (**Figure 5.25**) had also divided to the extent observed in *Lck*Cre control cells. 13% of CD4 negative cells remained undivided or divided more slowly, comparable to 8% in the case of cells obtained from *Lck*Cre controls. Therefore, *Lck*Cre *Ctnnb*$^{\Delta ex3}$ proliferated at comparable levels to *Lck*Cre control cells if Notch signaling was available.

In the complete absence of Notch signals, *Lck*Cre control cells did not differentiate or divide, or divided only slowly (95% of cells with high levels of CFSE). This was different in cells expressing stabilized β-catenin. As described above, these cells differentiated in the presence or absence of Notch signals to a greater extent than control cells. The CFSE signal detected in CD4 positive cells was low and thus similar to what was observed in control and *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells cultured on OP9-DL1. A greater percentage of differentiated cells however displayed higher CFSE signal intensity, indicating that cell division of differentiating cells was partially impaired in the absence of Notch signals. In fact, while only 13% of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells remained in an undivided state (higher levels of CFSE) in the presence of Notch signaling, this number was increased to 53% on OP9-GFP plus 1 µM GSI. These data indicate that β-catenin stabilization cannot overcome a partial requirement for Notch signaling for efficient

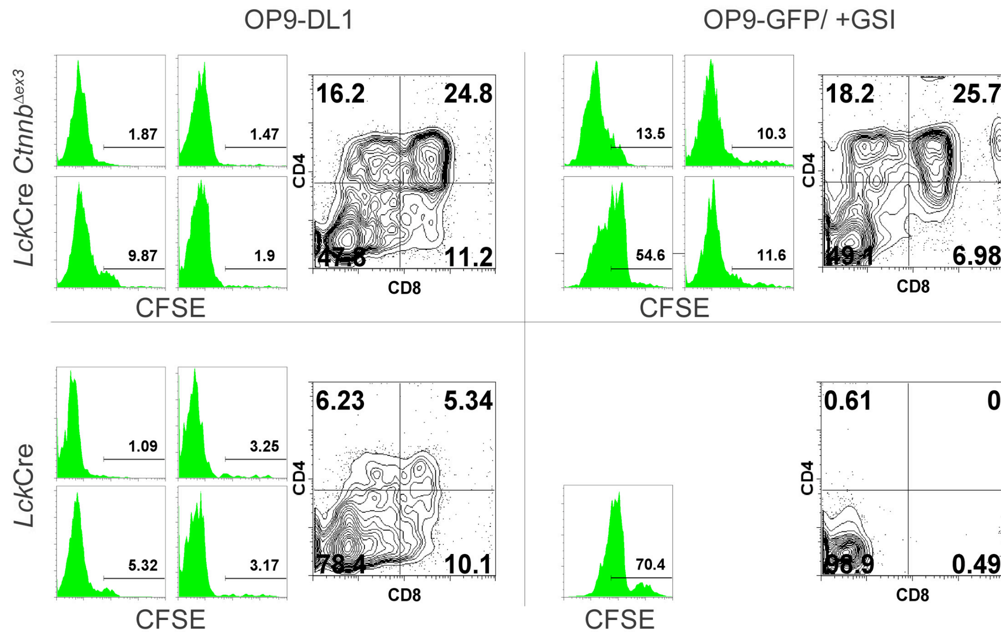


Figure 5.25: Impaired proliferation of DN cells in the absence of Notch signaling. Data shown were obtained in the same experiment as presented in **Figure 5.24.** To illustrate the proliferation of individual subsets more clearly, 4 histograms depicting CFSE intensity observed in the quadrants indicated in the surface profiles are shown. The 4 CFSE histograms are arranged in corresponding order to the adjacent quadrants in the FACS profiles showing CD4/ CD8 surface expression.

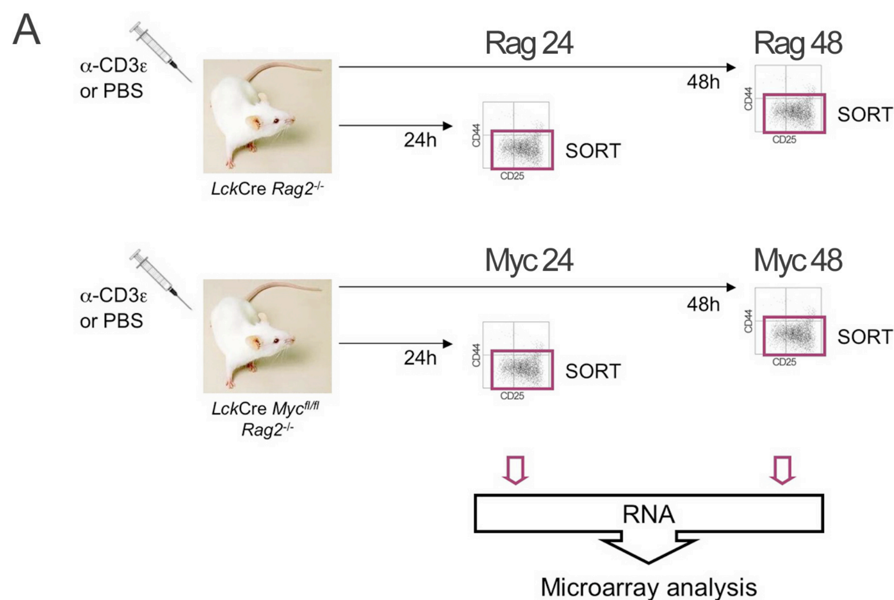
proliferation. Notch signaling might therefore be required for optimal proliferation after β-selection is completed.

Most of the cells that remained undivided appeared to be CD4 negative indicating that these cells might not be differentiated. Indeed, when DN, DP, CD4, and CD8 SP subsets were examined with regard to CFSE levels (**Figure 5.25**), it was obvious that cells with higher levels of CFSE were mostly contained within the DN subset. 55% of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ DN cells had higher levels of CFSE in the absence of Notch signaling versus 10% of DN cells with increased CFSE intensity in the DN compartment of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells cultured on OP9-DL1, and 5% in that of *Lck*Cre control cell cocultures on OP9-DL1. The other subsets contained cells with higher levels of CFSE, i.e. cells that had divided to a lesser extent at higher percentages as well, indicating that overall

proliferation was impaired in the absence of Notch signals, but was especially slow in the DN3 and DN4 cells constituting the DN subset in this experiment. It will therefore be of interest in the future to characterize the undividing DN subset with respect to TCRβ expression to establish the developmental status of the cells more clearly.

Taken together these data suggest that while β-catenin stabilization renders Notch signals dispensable for the DN to DP progression of immature thymocytes, and even accelerates this developmental transition, the differentiating cells are mostly devoid of TCRβ indicating that they did not receive preTCR signals. This in turn might suggest that β-catenin signaling normally occurs downstream of of β-selection to drive differentiation. Proliferation of cells that differentiate due to β-catenin activation is comparable to proliferation of differentiating control cells in the presence of Notch. In the absence of Notch however, it is slightly attenuated in differentiating cells, and a subset of DN cells remains undivided if Notch signaling is inhibited, indicating a tightly controlled interplay of Notch and Wnt/ β-catenin signaling at the preTCR checkpoint. Although over all few cells with β-catenin stabilization expressed preTCR, Notch had an effect on the occurrence of the subset of preTCR$^+$ cells, indicating that in the presence of Notch signaling preTCR$^+$ thymocytes with stabilized β-catenin had a developmental advantage. Moreover, these data indicate that Wnt and Notch signaling might mediate independent proliferation signals and that the developmental status of the cell matters with regard to the execution of these signals.

5.4 Global analysis of preTCR induced signaling events



B

Array #	Sample ID (Channel 1 – Cy3)		Sample ID (Channel 2 – Cy5)
1 A	Myc 0	X	Rag 0
1 B	Rag 0		Myc 0
2 A	Myc 24 PBS	X	Myc 24 CD3
2 B	Myc 24 CD3		Myc 24 PBS
3 A	Rag 24 PBS	X	Rag 24 CD3
3 B	Rag 24 CD3		Rag 24 PBS
4 A	Myc 48 PBS	X	Myc 48 CD3
4 B	Myc 48 CD3		Myc 48 PBS
5 A	Rag 48 PBS	X	Rag 48 CD3
5 B	Rag 48 CD3		Rag 48 PBS

Figure 5.26: Experimental design for global analysis of preTCR induced, proliferation independent gene expression changes. Myc = *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$, Rag = *Lck*Cre *Rag2*$^{-/-}$. **A**) Injection strategy: 10 mice per condition were injected with either a-CD3e or PBS and RNA was prepared from FACS sorted DN3 and DN4 cells (where applicable) 24 and 48 h post injection, resulting in pools of 5 mice per RNA preparation. **B**) Hybridization scheme for dye reversal strategy.

Our findings indicate that distinct signals exist downstream of of the preTCR that are responsible for proliferation *versus* differentiation and survival. Moreover, we demonstrated that c-Myc is required for proliferation at the preTCR checkpoint while being dispensable for developmental progression. In an attempt to identify additional signaling pathways that mediate differentiation or apoptosis in the absence of c-Myc mediated proliferation, we designed a microarray based screening approach (**Figure 5.26**).

Essentially, preTCR signals were induced by injecton of α-CD3ε antibodies or PBS into *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ (called "Myc" hereafter) and control *Lck*Cre *Rag2*$^{-/-}$ (called "Rag" hereafter) mice. DN3 and newly developing DN4 cells were then sorted 24 and 48 hours post injection and total RNA was prepared (**Figure 5.26A**). Five mice were pooled for each time point. Each sample was used to generate two probes carrying Cy3 and Cy5 fluorophore labels, respectively. Probes were hybridized to Agilent 4122F mouse whole genome gene chips (**Figure 5.26B**). Each array was hybridized with RNA obtained from α-CD3ε as well as from PBS injected animals to allow correction for background differences in gene expression. Using a dye reversal strategy to control for experimental variability, RNA from each time point (24 hours and 48 hours) for each genotype (Myc and Rag) was hybridized onto two separate chips. Data obtained from Myc and Rag mice were compared to distinguish between those genes that were similarly up- or downregulated in both mice and those genes that were differentially expressed after induction of preTCR signaling.

Preliminary analysis of the data thus obtained indicated, that 24 hours after α-CD3ε injection approximately 200 genes were similarly up- or downregulated more than 3-fold (approximately 75% of genes up and 25% of genes downregulated) in Myc as well as Rag mice (**Figure 5.27A**). Approximately 500 genes were differentially expressed between Myc and Rag mice (**Figure 5.27B**). These initial observations indicate that there are indeed changes in gene expression that depend on c-Myc (differentially expressed genes), and others that are independent of c-Myc (genes that are similarly regulated). Additional, more detailed analysis will aim at establishing genetic signatures of major

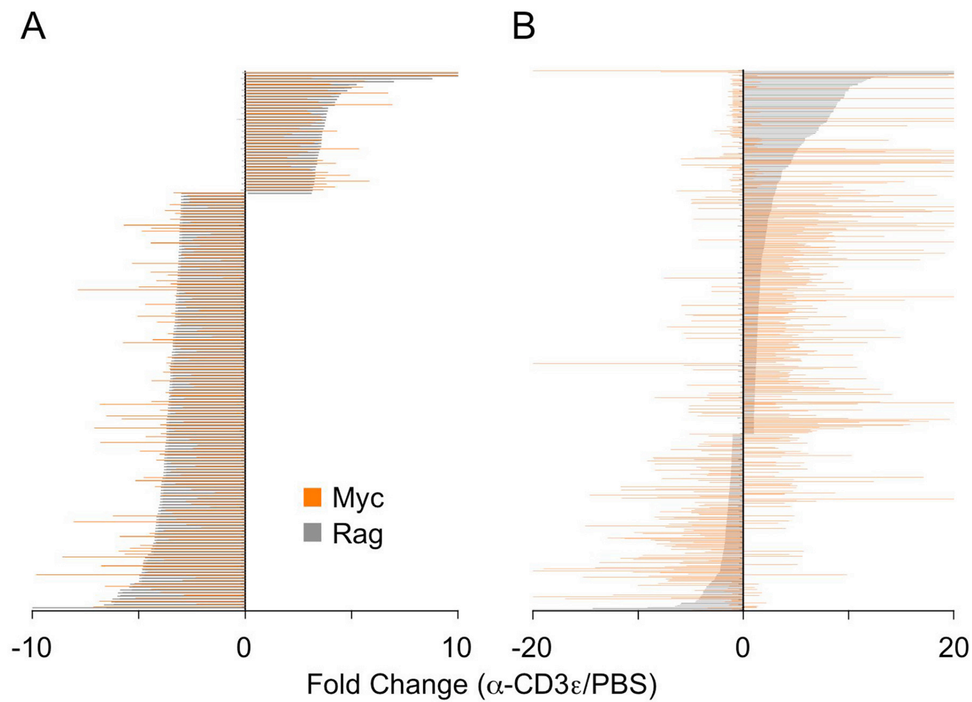


Figure 5.27: Expression changes 24 hours after α-CD3ε injection. Data were filtered based on a P-value of $P < 0.001$ as obtained from the dye-reversal comparison and a minimal intensity of $_{10}\log$ (Intensity)> 1.7. Genes were considered regulated if the fold change in expression was greater than 3 between α-CD3ε versus PBS injected groups. Data thus obtained were then grouped into **A**) genes that were similarly up- or downregulated, and **B**) genes that were differentially regulated between groups.

signaling pathways, including the Wnt/ β-catenin, Akt, and Notch (based on their downstream targets) cascades and their regulators. These analyses should provide further insight into the interdependence of signaling pathways at the β-selection checkpoint.

5.4.1 Generation of SciTetOβ for experimental validation

A proportion of the differentially expressed genes from the microarray analysis described before are expected to result from changes in signaling cascades that are not directly affecting gene expression but rather reflect changes in the activation status and/ or the stability of proteins. Naturally, such changes cannot be detected directly in a genetic screening approach. On the other hand, primary murine DN3 and DN4 cells are too limited in numbers to represent a feasible system for validation experiments. Therefore, we sought to establish a cell culture system that would allow the induction of preTCR signals, thus allowing initial biochemical target validation on the protein level using large numbers of cells that can be readily obtained. To this end, the TCRβ deficient and therefore preTCR negative cell line SciET27F was modified to express a TCRβ chain in a tetracycline dependent manner. The TCRβ chain was obtained from the construct that was used to generate TetOβ-LTH *Rag1*$^{-/-}$ mice as a 2.9 kb *Xho*I fragment containing 7 tetO sequences, a minimal CMV promoter, a rat β-globin intron, a TCRβ cDNA and a rat β-globin polyadenylation signal. This fragment

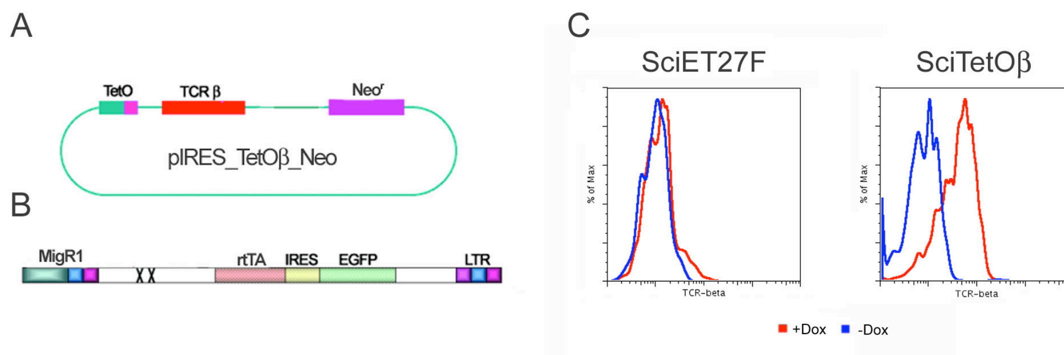


Figure 5.28: Inducible induction of preTCR in a cell culture system. A) Plasmid map of pIRES_TetOβ_Neo. **B**) Schematic representation of the MigR1-rtTA retrovirus. **C**) TCRβ surface expression was analyzed by FACS on the indicated cells that had ben treated with doxycycline o/n.

was ligated to a 3.3 kb fragment obtained from pIRES2-EGFP by sequential digest with *Ase*I and *Hpa*I. This fragment was devoid of the CMV promoter, the IRES, and the EGFP to obtain pIRES_TetOβ_Neo (**Figure 5.28A**). SciET27F cells were transfected with a pIRES_TetOβ_Neo, and selected with G418 (Neomycin equivalent) for stable integration of the construct into the host cell genome. Cells in which such an event had occurred were then infected with MigR1-rtTA retrovirus encoding the reverse tetracycline transactivator (rtTA) and EGFP (**Figure 5.28B**). $EGFP^+$, G418 resistant cells were sub-cloned and SciTetoβ was expanded from a single such clone.

Initial characterization of SciTetOβ cells showed that these cells do indeed express TCRβ in response to treatment with 1 μg/ ml doxycycline. SciET27F and SciTetOβ cells were cultured in the presence or absence of doxycycline o/n and stained for TCRβ surface expression. As depicted in **Figure 5.28C** SciTetOβ expressed TCRβ on their surface in response to doxycycline treatment as opposed to untreated SciTetOβ or SciET27F cells.

This convenient cell culture system represents an invaluable tool in the characterization of signaling events emanating from the preTCR.

6 Discussion

6.1 c-Myc mediates preTCR-induced proliferation but not developmental progression

PreTCR assembly and signaling promotes proliferation, survival, and differentiation of immature thymocytes at the $CD25^+CD44^-$ DN3 stage of development, essentially instructing them to the $\alpha\beta$ T cell lineage (von Boehmer, 1997). Using two mouse models that allow induction of the developmental $CD4^-$ $CD8^-$ DN to $CD4^+CD8^+$ DP transition, we found that an early event following the onset of preTCR signaling was the upregulation of c-Myc, a transcription factor involved in cell cycle regulation, cell growth, and apoptosis. Thus, within hours following induction of preTCR signaling, c-Myc protein levels increased indicating that this molecule was likely to play a role in the proliferation, survival, or differentiation processes mediated by preTCR signaling. We showed that conditional thymocyte specific ablation of c-Myc impaired cell growth and proliferation of immature thymocytes at the preTCR checkpoint. Despite reduced proliferation, the preTCR could still signal differentiation and survival to c-Myc deficient thymocytes both *in vivo* and *in vitro*. Our findings provide a dissection of preTCR signaling, and assign c-Myc specifically downstream of the proliferation but not the differentiation or survival signals.

Three lines of evidence support the notion that c-Myc is dispensable for the differentiation signals downstream of the preTCR. First, c-Myc deficient thymocytes progressed efficiently past the DN4 stage, although they yielded a reduced number of DP cells. Second, induction of preTCR like signaling by α-$CD3\varepsilon$ treatment of *Lck*Cre $Myc^{fl/fl}$ $Rag2^{-/-}$ mice promoted progression to the DP stage despite the c-Myc deficiency. Third, while control *Lck*Cre DN3 stage thymocytes cocultured on OP9-DL1 cells progressed to the DP stage while undergoing an average of five cell divisions, a substantial fraction of *Lck*Cre $Myc^{fl/fl}$ DN3 cells progressed to the DP stage and acquired $\alpha\beta$TCR surface expression with none to one cell division. These data demonstrate that the failure of c-Myc deficient thymocytes to proliferate does not impair their preTCR dependent differentiation potential. Our observations are in contrast with an

earlier report by Douglas and colleagues showing that c-Myc deficient thymocytes are unable to differentiate to the DP and SP stages in *Rag*$^{-/-}$*Myc*$^{-/-}$ chimeras (Douglas et al., 2001). More specifically, Douglas and colleagues generated chimeric animals from *Myc*$^{-/-}$ ES cells and *Rag1*$^{-/-}$ blastocysts in which the *Rag1*$^{-/-}$ cells cannot contribute to the lymphoid lineages. In this study *Myc*$^{-/-}$ progenitors populated embryonic thymi but had reduced proliferation and failed to develop beyond the late DN stages suggesting that c-Myc is essential for development past the preTCR checkpoint. This apparent discrepancy may be related to differences in the experimental systems. While Douglas and colleagues mainly focused on embryonic thymocytes with a constitutive c-Myc deficiency we conditionally ablated c-Myc immediately prior to the pre-TCR checkpoint at the CD25^{+}CD44^{+} DN2 to DN3 transition, thus avoiding the accumulation of defects from earlier developmental stages.

c-Myc ablation resulted in severely reduced DP numbers and overall decreased thymic cellularity. It is important to distinguish here between the potential of (even few) thymocytes to differentiate at all, and the number of cells observed at a given developmental stage. A reduction in a given subset does thus not necessarily mean that differentiation was impaired. Since c-Myc deficient thymocytes progressed to the DP stage without dividing while the equivalent *Lck*Cre cells reached this stage after undergoing four to five cell divisions, a ~32-fold reduction in the number of DP cells would be expected in *Lck*Cre Myc$^{fl/fl}$ mice. This prediction is in line with the reduction in the number of DP cells in *Lck*Cre Myc$^{fl/fl}$ mice. Similarly, fewer *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ thymocytes treated with α-CD3ε are expected to reach the DP stage due to decreased proliferation. Indeed, we detected a five-fold reduction in the number of DP thymocytes in α-CD3ε treated *Lck*Cre Myc$^{fl/fl}$ *Rag2*$^{-/-}$ mice as compared to *Rag2*$^{-/-}$ controls four days after injection. Our findings that c-Myc deficiency does not impair differentiation are in line with recent reports showing that *in vitro* cultured, c-Myc deficient HSC are able to differentiate along the myeloid and lymphoid lineages (Wilson et al., 2004). Interestingly, in the case of the HSC, as in thymocytes, this occurs in the absence of significant proliferation. Although our data show that in

the absence of c-Myc differentiation is in principle not inhibited *in vivo*, the reduced numbers of terminally differentiated cells might give the impression of a developmental block, and have devastating consequences with respect to immune function in the host. So far *Lck*Cre Myc$^{fl/fl}$ mice were kept under specifically pathogen free conditions, i.e. in an unusually clean environment. Moreover, we did not yet address the peripheral T cell compartment with respect to function. *Lck*Cre Myc$^{fl/fl}$ mice have very few peripheral T cells (data not shown). It has long been described that c-Myc is regulated by multiple mechanisms in response to T cell activation (Lindsten et al., 1988), and more recently it was found that transgenic overexpression of the c-Myc antagonist Mad-1 impaired proliferation in response to T cell activation by mitogens (Iritani et al., 2002). c-Myc deficiency is therefore expected to negatively impact the ability of *Lck*Cre Myc$^{fl/fl}$ mice to react to challenges of the immune system, but this remains to be determined in the future.

The c-Myc deficient DN4 stage thymocytes were cycling at lower frequencies, and were smaller, indicating that the proliferative signals attributed to the preTCR were impaired in the absence of c-Myc. This proliferative block was detected both *in vivo* and *in vitro* but was more dramatic in OP9-DL1 stromal cell cocultures seeded with sorted thymocytes from either the DN3 or the DN4 stage. c-Myc deficient thymocytes failed to undergo more than one cell division in these cultures. The mechanism by which c-Myc promotes thymocyte proliferation may rely on the regulation of cell cycle inhibitors such as p27^{Kip}, Gadd45α and p21^{Cip1} (Claassen and Hann, 2000; Coller et al., 2000; Gartel et al., 2001; Mitchell and El-Deiry, 1999; Mukherjee and Conrad, 2005). This is indicated by the elevated expression of these molecules in c-Myc deficient thymocytes. Elevated levels of p27^{Kip} have previously been proposed to result in proliferation defects of c-Myc deficient B-cells (de Alboran et al., 2001). Gadd45α has been suggested to determine the susceptibility of a cell to p21^{Cip1} induced cell cycle arrest (Zhang et al., 2003), has been linked to T-cell proliferation (Salvador et al., 2002), and has also been reported to suppress cell growth via inhibition of the G2-M promoting

Cdc2 kinase (Jin et al., 2000).
Interestingly, both message and protein levels of the growth promoting Cyclin D3 appeared unchanged. This is noteworthy considering that Cyclin D3 has previously been reported to control the proliferative expansion of late stage DN and ISP thymocytes (Sicinska et al., 2003). This observation indicates therefore that Cyclin D3 mediated proliferation cannot compensate for c-Myc deficiency, suggesting that the preTCR triggers multiple proliferation pathways. We cannot exclude the possibility that the regulation of Cyclin D3 relies on protein modifications that are not detectable by the methods we used for analysis, and it remains a possibility that c-Myc does affect Cyclin D3 mediated proliferation.

c-Myc has been shown to promote cell growth in B cells (Iritani and Eisenman, 1999; Schuhmacher et al., 1999) and thymocytes (Iritani et al., 2002). By contrast, Trumpp and colleagues observed that reduced levels of c-Myc do not affect the size of T cells upon activation (Trumpp et al., 2001). Our finding that c-Myc ablation at the DN3 stage of thymocyte development resulted in small cells that failed to develop into blasts at the DN4 stage suggests that while reduced levels of c-Myc may still be sufficient to mediate cell growth a complete ablation is not. This explanation is in line with recent findings that c-Myc is likely to regulate cellular growth through ribosome biogenesis (Arabi et al., 2005; Grandori et al., 2005; Grewal et al., 2005), and that this regulation requires only low levels of c-Myc in the nucleolus.

Our data indicate that the reduced thymic cellularity observed in *Lck*Cre Myc$^{fl/fl}$ mice is most likely the result of impaired proliferation but not reduced survival. However, c-Myc has long been thought to sensitize cells to apoptosis particularly when it is overexpressed. On the other hand, conditional ablation of c-Myc was shown not to affect the survival of HSC (Wilson et al., 2004) or primary B lymphocytes (de Alboran et al., 2004). In line with these observations, we found that spontaneous apoptosis of immature c-Myc deficient thymocytes was comparable to that of control thymocytes at the equivalent stages. Moreover, we

did not detect deregulated expression of several genes implicated in cell survival/ death that have been classified as potential c-Myc target genes, such as p53, Bcl-2, and Bcl-x_L (Fernandez et al., 2003; Reisman et al., 1993; Tavtigian et al., 1994).

In summary, by ablating c-Myc expression in thymocytes at the β-selection checkpoint we were able to show that proliferation downstream of the preTCR was indeed mediated by this transcription factor. Moreover, since differentiation and apoptosis were unperturbed in the absence of c-Myc mediated proliferation, we hypothesize that proliferation is an independent event in response to preTCR signals. Here we show that c-Myc is rapidly induced upon activation of the preTCR. This could be directly controlled by preTCR signals or it could be an indirect consequence. However, the rapid upregulation of c-Myc protein levels within 6 hours after induction of the preTCR argues in favor of a direct control of c-Myc by the preTCR. Irrespective of the mechanism by which preTCR induces c-Myc our study reveals for the first time a bifurcation of signaling pathways at the preTCR checkpoint and shows that differentiation of thymocytes occurs efficiently in the absence of c-Myc dependent proliferation.

6.2 Akt1 cannot compensate for Notch signals in adult thymocytes

Having established that differentiation and survival were not impaired in the absence of c-Myc, we next thought to examine other signaling cascades that might be activated at the β-selection checkpoint. The phosphatidylinositol-3 (PI(3)) kinase pathway may be involved in β-selection. Earlier studies have shown that ablation of PI(3) kinase γ inhibits the DN to DP transition in the thymus (Rodriguez-Borlado et al., 2003; Sasaki et al., 2000). Likewise, genetic ablation of the phosphoinositide-dependent kinase 1 (PDK1), that is rate-limiting for many PI(3) kinase dependent pathways, results in a developmental block at the DN stage of thymocyte development (Hinton et al., 2004). The growth-factor-

regulated serine/ threonine kinase Akt/ Protein kinase B (PKB) is an example for a kinase that is activated by PDK1 phosphorylation. The Akt family consists of Akt1, 2, and 3, which appear to be central players in regulation of metabolism, cell survival, motility, transcription and cell-cycle progression (Downward, 1998; Fayard et al., 2005). Apart from phosphorylating and inhibiting pro-apoptotic mediators such as Bad, FOXO family members, and IκB kinase-β (IKK-β) (Datta et al., 1997), the survival signal mediated by Akt is thought to result in part from its regulatory function in glucose metabolism (Gottlob et al., 2001; Rathmell et al., 2003). Indeed, one study implies that Notch signaling promotes survival of immature thymocytes at the preTCR checkpoint by a mechanism that involves activated Akt and increased glucose metabolism (Ciofani and Zuniga-Pflucker, 2005). However, a molecular connection between the Notch and Akt signaling pathways has not been established so far. Akt activation mostly depends on cell-extrinsic signals transmitted by growth factor and cytokine receptors. Thus, it appears likely that Notch induced activation of Akt is an indirect event. Moreover, observations presented here are in contrast with these findings as we show that expression of a constitutively active form of Akt (MyrAkt) in mice does not enable adult DN3 stage thymocytes to overcome the need for Notch signals on OP9-GFP cocultures. The MyrAkt cDNA used to generate MyrAkt mice is identical to the one used in the cited study. However, these investigators used retroviral delivery to achieve MyrAkt expression in cells that were derived from the fetal liver on *ex vivo* cocultures. Fetal T cell development differs from that in adult mice in multiple aspects, such as the generation of different repertoires of γδ T cells (Carding and Egan, 2002). In addition, commitment to the T lineage in the embryo appears to occur in the absence of bi-potent T/B lineage progenitors (Douagi et al., 2002; Kawamoto et al., 2000) and independent of Notch signaling (Harman et al., 2005; Masuda et al., 2005). Similarly, fetal liver derived DN3 cells may respond differently to Notch signals than adult DN3 cells. It is also possible that the expression levels of the activated Akt are different in retrovirally transduced *Rag*$^{-/-}$ DN3 cells versus DN3 cells obtained from MyrAkt mice by cell sorting.

While we were unable to confirm a role for Akt in mediating Notch survival signals in adult DN thymocytes, our data do indicate that Akt is activated downstream of the preTCR, as we observe increased p-Akt (Thr308) levels in preTCR$^+$ SCB.29 compared to preTCR$^-$ SciET27F cell lines. Indeed, these findings were confirmed in preTCR inducible mice as well as in *Rag*$^{-/-}$ mice stimulated by α-CD3ε injection (data not shown) (Mao et al., 2007). By genetic ablation of different combinations of Akt1, 2 and 3 these investigators further confirmed a role for Akt1 and 2 and to a lesser extent for Akt3 in the DN to DP transition. Moreover, when the MyrAkt transgene was introduced into *Rag*$^{-/-}$ mice it promoted the development of DP cells, indicating that Akt1 can mimic preTCR signaling to some extent. In fact, a different study has shown that ablation of the PI(3) kinase pathway regulatory phosphatase and tensin homologue deleted on chromosome 10 (Pten) promotes thymocyte development in the absence of IL7-R as well as preTCR in mice (Hagenbeek et al., 2004). Pten normally counteracts the PI(3) kinase pathway, and its ablation therefore leads to increased activation of Akt and other downstream of targets of PI(3) kinase signaling (Seminario and Wange, 2003). The authors conclude, however, that Akt is not the only mediator of the effect they observe, and discuss the possible involvement of the small GTPases Rac and Rho, which are influenced by PI(3) kinase (Reif et al., 1996) and have been shown to affect growth of early T cell precursors (Galandrini et al., 1997; Gomez et al., 2000).

Therefore, our data are in line with a partial role for Akt signaling in thymocyte development, most likely downstream of preTCR signals. Akt1 expression in mice, however, did not allow adult DN thymocytes to progress to the DP stage in the absence of Notch signaling, indicating that ectopic expression of activated Akt cannot compensate for the absence of Notch signaling in the adult mouse.

6.3 Enforced Wnt signaling overcomes Notch requirement at the β-selection checkpoint

Another major signaling pathway that has been implicated in the development of multiple organ systems is the Wnt/ β-catenin cascade. As detailed before, multiple studies suggest that this cascade has a critical role in thymocyte development (Gounari et al., 2001; Gounari et al., 2005; Ioannidis et al., 2001; Okamura et al., 1998; Pongracz et al., 2006; Staal et al., 2001; Weerkamp et al., 2006a; Xu et al., 2003). Stabilization of β-catenin in the thymus has previously been shown to promote development of DN thymocytes to the DP stage in the absence of preTCR signaling (Gounari et al., 2001) indicating that it must convey a strong differentiation signal to thymocytes overcoming a checkpoint that is normally highly restrictive. Another absolute requirement for the developmental transition of DN3 thymocytes to the DP stage is Notch signaling (Maillard et al., 2006). Therefore, it was of interest to establish whether β-catenin stabilization could also overcome the need for Notch signals at the preTCR checkpoint. To answer this question OP9-DL1 and OP9-GFP cocultures were employed, representing presence and absence of Notch signals, respectively. Data presented here indicate that the stabilization of β-catenin at the DN2 to DN3 stage transition enables thymocytes to develop in the complete absence of Notch signals. Such, sorted DN3 thymocytes from *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice develop to the DP stage on OP9-GFP cocultures just as well as they do on OP9-DL1 feeder cells. We can also exclude the possibility that residual expression of other Notch ligands on OP9 cells aided this developmental progression. This is because thymocytes with stabilized β-catenin progressed to the DP stage on OP9-DL1 and OP9-GFP cocultures even after inhibition of IC-Notch formation through treatment with γ-secretase inhibitor (GSI). These findings indicate that Notch signaling was dispensable for *ex vivo* developmental progression of cells that express a stabilized form of β-catenin.

Another potential explanation for this Notch independence could be that *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice harbor *Notch* mutations, resulting in the expression of activated

Notch. We can, however, exclude this possibility as well since previous observations indicate that β-catenin stabilization results in transcriptional downregulation of Notch signaling components, such as Notch1, Hes-1, and Deltex-1, as well as the positive regulator of Notch signaling Lunatic Fringe (Gounari et al., 2005; Guo et al., 2007). Moreover, sequencing of the *Notch1* gene in cells obtained from β-catenin induced lymphomas established the integrity of exons 26 and 27, encoding the heterodimerization domain, and exon 34 encoding the PEST domain of the Notch1 protein (Guo et al., 2007). Both regions are frequently found mutated in T-ALL samples leading to oncogenic Notch activation (Weng et al., 2004).

As opposed to *Lck*Cre control cells only a small fraction of DP *Lck*Cre *Ctnnb*$^{\Delta ex3}$ cells that had been differentiated from DN3 cells on OP9-LD1 cocultures showed TCRαβ surface expression. This observation was even more pronounced if Notch signaling was inhibited or absent. Interestingly, the gene encoding the pTα chain, *Ptcra*, is upregulated in murine thymoma cell lines upon retroviral transduction with IC-Notch (Deftos et al., 2000). It was demonstrated that the *Ptcra* promoter contains a CSL binding site and could be activated by IC-Notch *in vitro* (Reizis and Leder, 2002). This might be a possible mechanism by which Notch signaling might regulate the expression of preTCR. However, the physiological role of Notch signaling for pTα expression remains controversial, as pTα expression was not affected in mice upon conditional deletion of Notch1 from the DN3 stage onwards (Wolfer et al., 2002), and was not found upregulated upon Notch signal induction in immature human thymocytes in a microarray screen (Weerkamp et al., 2006b). Another mechanism that would result in low or absent TCRαβ surface expression while preTCR levels are not perturbed (but cannot be detected directly by FACS) is impaired TCRα rearrangement or expression. In fact while TCRβ expression was unperturbed, TCRα gene transcription was severely reduced in mice lacking both the β-catenin dependent transcription factors Tcf1 and Lef1 (Okamura et al., 1998).

To exclude the possibility that preTCR is required at low levels that might not result in the expression of mature αβTCR or that the observed effects are due to defective TCRα rearrangements, *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ mice were studied. In the absence of *Rag* mice cannot recombine the TCR genes and therefore lack TCRβ expression resulting in preTCR deficiency and a developmental block at the DN3 stage in the thymus. The expression of stabilized β-catenin can overcome this block, but DP cells developing in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ mice are preTCR^{-} (Gounari et al., 2001). The data presented here clearly show that progression of preTCR^{-} DN thymocytes to the DP stage is possible *ex vivo* in the presence of stabilized β-catenin, and interestingly, that this transition does not depend on Notch signals. Thus, *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ DN3 cells gave rise to DP cells with equal efficiency on OP9-DL1 as well as OP9-GFP feeder cells, in the absence as well as the presence of GSI. Consequently, we established that even in the complete absence of two key signaling pathways at the β-selection checkpoint, preTCR and Notch signaling, β-catenin stabilization enables developing thymocytes to differentiate *ex vivo*.

So far, Akt is the only other molecule described leading to developmental progression of DN3 stage thymocytes in the absence of both Notch1 and preTCR (Ciofani and Zuniga-Pflucker, 2005). As discussed above, the data presented here are not in agreement with a role for Akt downstream of Notch. They do, however, support the notion that Akt is activated upon preTCR signaling. It is therefore tempting to speculate about a role for Akt upstream or downstream of β-catenin in response to preTCR signaling (**Figure 6.1**). It has recently been described that Akt directly phosphorylates β-catenin at Ser552, releasing it from the cell-cell contacts and resulting in stabilization of cytosolic β-catenin, ultimately leading to increased transcriptional activity (Fang et al., 2007; He et al., 2007). In addition to this, a recent study also describes the identification of nine putative Tcf/ Lef-binding elements in the *AKT1* gene in humans, and identifies β-catenin mediated AKT1 activation as a likely mechanism in the etiology of colorectal cancer (Dihlmann et al., 2005). Moreover, a regulatory role for Akt in Wnt

signaling has previously been proposed (Fukumoto et al., 2001). According to this study, activated Akt can be found in complex with GSK-3β and Axin in the presence of Dishevelled (i.e. upon Wnt signaling). Akt phosphorylates GSK-3β at Ser9 impairing GSK-3β mediated β-catenin phosphorylation, which in turn leads to accumulation of nuclear β-catenin. Together with the likewise observed induction of Akt upon Wnt signaling this results in a positive feedback loop of

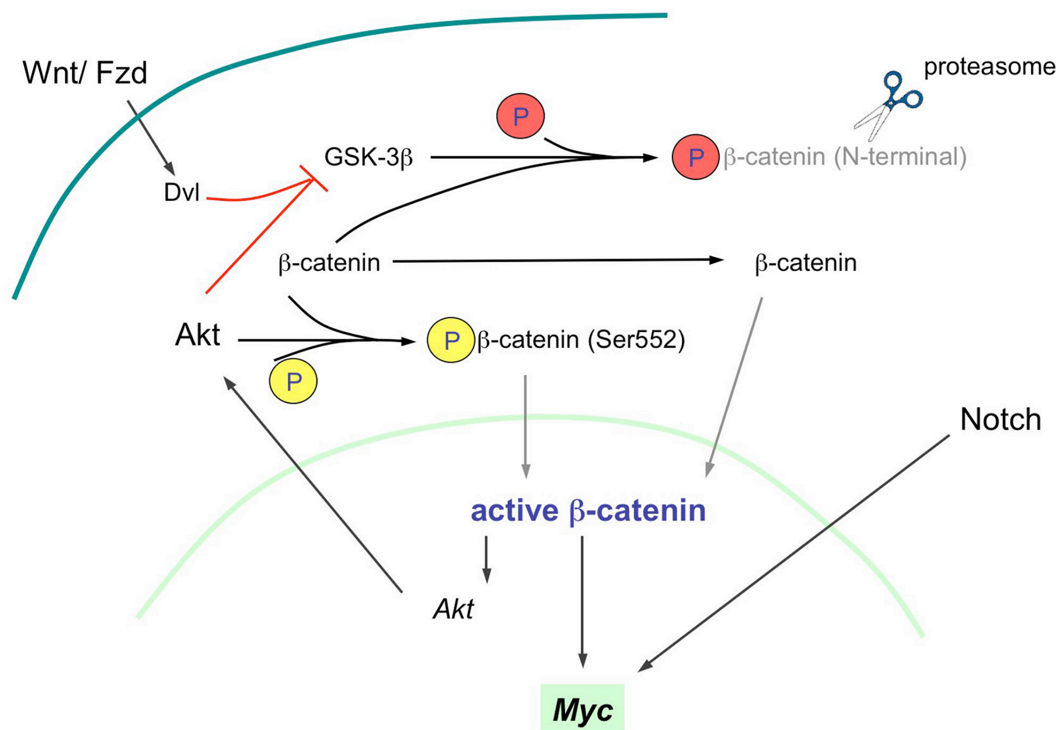


Figure 6.1: Interplay between Akt, Wnt/ β-catenin and Notch signaling pathways, resulting in the activation of *Myc* transcription. In the absence of Wnt signaling β-catenin is degraded via the proteasome. This pathway is inhibited upon Wnt/ Fzd interaction resulting in the activation of Dvl. In the presence of Dvl, Akt phosphorylation additionally inhibits GSK-3β. Active β-catenin accumulation in the nucleus leads to Tcf/ Lef mediated transctiptional activation of *Akt* and *Myc*. By a different mechanism, Akt can also phosporylate β-catenin at Ser552, resulting in increased stabilization and translocation to the nucleus. c-Myc has been described as a direct Notch target. Dvl: Dishevelled, Fzd: Frizzled

activated Wnt signaling components. Akt only phosphorylates complex bound GSK-3β in the presence of Dishevelled, providing an explanation for why GSK-3β phosphorylation is not observed when Akt is activated via the PI(3) kinase pathway, in the absence of Wnt signals (and therefore Dishevelled) (Chen et al., 2000; Ding et al., 2000). The role of this signaling loop in thymocytes remains to be addressed, but it provides an interesting hypothetical mechanism by which preTCR mediated Akt activation could sustain canonical Wnt signaling. This model (**Figure 6.1**) also implies a possible mechanism by which initially independent maturation signals obtained by immature thymocytes at the DN to DP transition might be integrated to result in Tcf/ Lef mediated gene activation.Our data also suggest, that the stabilization of β-catenin accelerates differentiation, especially in the absence of preTCR. Thus, we observed that the developmental progression of *Lck*Cre $Ctnnb^{\Delta ex3}$ cells on OP9-DL1 feeder cells was considerably faster than that of *Lck*Cre cells. *Lck*Cre $Ctnnb^{\Delta ex3}$ $Rag2^{-/-}$ DN3 cells progressed even faster. These observations prompt the intriguing hypothesis that β-selection might provide signals setting a “slow pace” of differentiation, a useful signal considering that TCRβ rearrangement is normally occurring at this stage, and that rapid cell cycle progression in parallel to DNA rearrangement would likely result in cancer. β-catenin, on the other hand, may drive rapid differentiation after the preTCR checkpoint. Some evidence for a delayed function of β-catenin with respect to Notch signaling at the β-selection checkpoint comes from the observation that β-catenin stabilization downregulates Notch and Notch downstream mediators as well as Notch signaling regulators (Gounari et al., 2005; Guo et al., 2007). In this context, another study showing that the Notch inhibitor Numb has a Tcf/ Lef binding site in its promoter and inhibits the Notch/ Wnt signaling axis is also of interest (Katoh, 2006). In addition, recent evidence indicates that DN3 cells that have undergone β-selection (DN3b) are no longer dependent on Notch signals for differentiation (Taghon et al., 2006). In this context our data indicate that Wnt/ β-catenin signaling might be activated after β-selection to drive the differentiation of such DN3b cells.

To establish a role for β-catenin specifically after β-selection it will be of high interest to sort DN3b, i.e. DN3 cells that have completed β-selection, and examine their developmental progression in OP9-DL1 cocultures in the presence of different small molecule or retrovirally encoded (such as Dkk1) inhibitors of Wnt/ β-catenin signaling. These experiments should answer whether Wnt/ β-catenin signaling is necessary downstream of the preTCR for differentiation to the DP stage.
One observation presented here would, however, argue against a complete dispensability of Notch signals post β-selection. Namely, in the absence of Notch signals proliferation of cells with stabilized β-catenin is reduced, especially in a fraction of undifferentiated DN cells, but also to a lesser extent in differentiated cells. These data indicate that β-catenin stabilization cannot overcome a partial requirement for Notch signaling for efficient proliferation. Notch signaling might therefore be required for optimal proliferation after β-selection is completed. Indeed there exists evidence for Notch dependent proliferation of DN3b and DN4 cells from OP9 coculture experiments (Garbe et al., 2006; Taghon et al., 2006) as well as for the loss of Notch dependence of differentiation at some point during this developmental progression (Taghon et al., 2006).

Given our data detailing a role for c-Myc in proliferation of DN4 stage thymocytes, as well as the observation that β-catenin activation as well as Notch appear to transmit proliferation signals, it is highly interesting to note that c-Myc has now been described as a target of both of these pathways. Studies of c-Myc in the intestine have established the Wnt signaling pathway as an upstream effector a while ago (He et al., 1998; van de Wetering et al., 2002b). Very recently, c-Myc was also shown to mediate most of the target gene inductions following the loss of the adenomatous polyposis coli tumor suppressor protein (APC) that results in constitutive activation of β-catenin (Sansom et al., 2007). Also recently, Notch1 was found to bind to the human *Myc* promoter and to affect transcription of this gene (Palomero et al., 2006). In addition, a conserved CSL consensus sequence was found in the *Myc* gene, and it was shown that DN3

cells depend on Notch signals to maintain c-Myc expression (Weng et al., 2006). It is therefore tempting to hypothesize that c-Myc might be an integrator of proliferation signals received by DN cells from individual signals around the β-selection checkpoint.

Further support for an integration of proliferation signals at the c-Myc level comes from a comparison of the molecular requirements for the development of leukemia as a result of deregulated expression of Notch and β-catenin. Notch activating mutations were described in over 50% of the examined cases of human T-ALL (Weng et al., 2004), and Notch appears to be a key player in mouse models of T-cell leukemia as well. Transgenic expression of activated Notch1 (Pear et al., 1996) or Notch3 (Bellavia et al., 2000) mediates thymocyte transformation that is linked with the modulation of pre-TCR signaling, inhibition of the E2A pathway and upregulation of c-Myc (Zweidler-McKay and Pear, 2004). c-Myc is found upregulated in a variety of malignancies and in particular those of hematopoietic origin, and may be the common denominator in the etiology of leukemia (Ferrando et al., 2002; Hoffman et al., 2002). The upregulation of c-Myc expression was a consistent secondary event in lymphomas originating from the stabilized expression of β-catenin (Guo et al., 2007). The β-catenin induced lymphoma does not require or select for Notch activation, and it is one possible explanation that this results from the Notch independent upregulation of c-Myc in this model.

*Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice develop lymphomas, and it would therefore be possible that the observed differentiation in the absence of developmental cues otherwise required for the progression of T cell development in wt mice reflects malignant transformation of *Lck*Cre *Ctnnb*$^{\Delta ex3}$ DN3 cells prior to cocultures. This is highly unlikely, however, since the lymphomas developing in *Lck*Cre *Ctnnb*$^{\Delta ex3}$ mice occur around 3-5 months of age and are unanimously preTCR positive (Guo et al., 2007). Not only were the experiments described in this study performed with thymocytes obtained from mice around 6 weeks of age, which is clearly prior to the occurrence of lymphomas, but the developing DP cells were mostly preTCR

deficient, suggesting that these cells were not transformed. In addition to this, differentiation in the absence of Notch was also observed for DN3 cells from *Lck*Cre *Ctnnb*$^{\Delta ex3}$ *Rag2*$^{-/-}$ mice, and these animals do not develop lymphomas at all.

In conclusion, data presented in this study provide insight into the molecular mechanisms governing the mediation of survival, proliferation and differentiation downstream of the preTCR checkpoint. Our work indicates that proliferation can be uncoupled from survival and differentiation of immature thymocytes. However, we also presented evidence that the signaling mechanisms mediating these processes might be interdependent and regulate each other in a complex network. In the future we hope to further address the signaling requirements during β-selection by more detailed analysis of our microarray data, comparing the effects of preTCR signaling in the absence and/ or presence of c-Myc mediated proliferation. Since Notch, Akt, and Wnt signaling appear to be major players at the DN to DP transition, our analysis will focus on these pathways and their regulators. Because many upstream events in these signaling cascades rely on protein modifications, such as changes in the phosphorylation status, the conformation, or localization, rather than on changes in mRNA expression levels, we will analyze the array data with respect to expression changes of target genes and effectors of these mediators. *Hes-1* and *Notch1* itself have been described as Notch targets (Weerkamp et al., 2006b). Several gene families have been implicated to mediate survival signals of Akt, for example NFAT and the FOXO family of Forkhead transcription factors (Fruman, 2004). We are hoping to obtain a number of candidate genes using this screening approach. These genes can subsequently be validate in the preTCR inducible cell line SciTetOβ or in *ex vivo* coculture of immature primary thymocytes. Future research will involve modulating the expression of important candidate genes using genetic approaches or retroviral manipulation.

7 References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J *16*, 3797-3804.

Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S. E. (2001). Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659-669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A., et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295-306.

Aifantis, I., Azogui, O., Feinberg, J., Saint-Ruf, C., Buer, J., and von Boehmer, H. (1998). On the role of the pre-T cell receptor in alphabeta versus gammadelta T lineage commitment. Immunity *9*, 649-655.

Aifantis, I., Borowski, C., Gounari, F., Lacorazza, H. D., Nikolich-Zugich, J., and von Boehmer, H. (2002). A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. Nat Immunol *3*, 483-488.

Aifantis, I., Gounari, F., Scorrano, L., Borowski, C., and von Boehmer, H. (2001). Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-kappaB and NFAT. Nat Immunol *2*, 403-409.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Allman, D., Sambandam, A., Kim, S., Miller, J. P., Pagan, A., Well, D., Meraz, A., and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. Nat Immunol *4*, 168-174.

Anderson, G., and Jenkinson, E. J. (2001). Lymphostromal interactions in thymic development and function. Nat Rev Immunol *1*, 31-40.

Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., et al. (2005). c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat Cell Biol *7*, 303-310.

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Ausserlechner, M. J., Obexer, P., Bock, G., Geley, S., and Kofler, R. (2004). Cyclin D3 and c-MYC control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. Cell Death Differ *11*, 165-174.

Badiani, P. A., Kioussis, D., Swirsky, D. M., Lampert, I. A., and Weston, K. (1996). T-cell lymphomas in v-Myb transgenic mice. Oncogene *13*, 2205-2212.

Bain, G., Cravatt, C. B., Loomans, C., Alberola-Ila, J., Hedrick, S. M., and Murre, C. (2001). Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. Nat Immunol *2*, 165-171.

Balciunaite, G., Ceredig, R., Fehling, H. J., Zuniga-Pflucker, J. C., and Rolink, A. G. (2005). The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. Eur J Immunol *35*, 1292-1300.

Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science *280*, 596-599.

Bellavia, D., Campese, A. F., Alesse, E., Vacca, A., Felli, M. P., Balestri, A., Stoppacciaro, A., Tiveron, C., Tatangelo, L., Giovarelli, M., et al. (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. Embo J *19*, 3337-3348.

Bender, T. P., Kremer, C. S., Kraus, M., Buch, T., and Rajewsky, K. (2004). Critical functions for c-Myb at three checkpoints during thymocyte development. Nat Immunol *5*, 721-729.

Benz, C., Heinzel, K., and Bleul, C. C. (2004). Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. Eur J Immunol *34*, 3652-3663.

Besseyrias, V., Fiorini, E., Strobl, L. J., Zimber-Strobl, U., Dumortier, A., Koch, U., Arcangeli, M. L., Ezine, S., Macdonald, H. R., and Radtke, F. (2007). Hierarchy of Notch-Delta

interactions promoting T cell lineage commitment and maturation. J Exp Med *204*, 331-343.

Bhandoola, A., and Sambandam, A. (2006). From stem cell to T cell: one route or many? Nat Rev Immunol *6*, 117-126.

Borowski, C., Li, X., Aifantis, I., Gounari, F., and von Boehmer, H. (2004). Pre-TCRalpha and TCRalpha are not interchangeable partners of TCRbeta during T lymphocyte development. J Exp Med *199*, 607-615.

Buckley, A. F., Kuo, C. T., and Leiden, J. M. (2001). Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc--dependent pathway. Nat Immunol *2*, 698-704.

Cairo, S., De Falco, F., Pizzo, M., Salomoni, P., Pandolfi, P. P., and Meroni, G. (2005). PML interacts with Myc, and Myc target gene expression is altered in PML-null fibroblasts. Oncogene *24*, 2195-2203.

Campese, A. F., Garbe, A. I., Zhang, F., Grassi, F., Screpanti, I., and von Boehmer, H. (2006). Notch1-dependent lymphomagenesis is assisted by but does not essentially require pre-TCR signaling. Blood *108*, 305-310.

Carding, S. R., and Egan, P. J. (2002). Gammadelta T cells: functional plasticity and heterogeneity. Nat Rev Immunol *2*, 336-345.

Ceredig, R., Lowenthal, J. W., Nabholz, M., and MacDonald, H. R. (1985). Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. Nature *314*, 98-100.

Chan, A. C., Dalton, M., Johnson, R., Kong, G. H., Wang, T., Thoma, R., and Kurosaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. Embo J *14*, 2499-2508.

Chen, R. H., Ding, W. V., and McCormick, F. (2000). Wnt signaling to beta-catenin involves two interactive components. Glycogen synthase kinase-3beta inhibition and activation of protein kinase C. J Biol Chem *275*, 17894-17899.

Ciofani, M., Knowles, G. C., Wiest, D. L., von Boehmer, H., and Zuniga-Pflucker, J. C. (2006). Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. Immunity *25*, 105-116.

Ciofani, M., Schmitt, T. M., Ciofani, A., Michie, A. M., Cuburu, N., Aublin, A., Maryanski, J. L., and Zuniga-Pflucker, J. C. (2004). Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. J Immunol *172*, 5230-5239.

Ciofani, M., and Zuniga-Pflucker, J. C. (2005). Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. Nat Immunol *6*, 881-888.

Claassen, G. F., and Hann, S. R. (2000). A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. Proc Natl Acad Sci U S A *97*, 9498-9503.

Coller, H. A., Grandori, C., Tamayo, P., Colbert, T., Lander, E. S., Eisenman, R. N., and Golub, T. R. (2000). Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci U S A *97*, 3260-3265.

Colucci, F., and Di Santo, J. P. (2000). The receptor tyrosine kinase c-kit provides a critical signal for survival, expansion, and maturation of mouse natural killer cells. Blood *95*, 984-991.

Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R., and Dale, T. C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. Embo J *15*, 4526-4536.

Dang, C. V. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol *19*, 1-11.

Daniels, D. L., and Weis, W. I. (2005). Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol *12*, 364-371.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev *7*, 671-682.

de Alboran, I. M., Baena, E., and Martinez, A. C. (2004). c-Myc-deficient B lymphocytes are resistant to spontaneous and induced cell death. Cell Death Differ *11*, 61-68.

de Alboran, I. M., O'Hagan, R. C., Gartner, F., Malynn, B., Davidson, L., Rickert, R., Rajewsky, K., DePinho, R. A., and Alt, F. W. (2001). Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity *14*, 45-55.

Deftos, M. L., and Bevan, M. J. (2000). Notch signaling in T cell development. Curr Opin Immunol *12*, 166-172.

Deftos, M. L., Huang, E., Ojala, E. W., Forbush, K. A., and Bevan, M. J. (2000). Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. Immunity *13*, 73-84.

Dihlmann, S., Kloor, M., Fallsehr, C., and von Knebel Doeberitz, M. (2005). Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. Carcinogenesis *26*, 1503-1512.

Ding, V. W., Chen, R. H., and McCormick, F. (2000). Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. J Biol Chem *275*, 32475-32481.

Dooley, J., Erickson, M., Gillard, G. O., and Farr, A. G. (2006). Cervical thymus in the mouse. J Immunol *176*, 6484-6490.

Dose, M., Khan, I., Guo, Z., Kovalovsky, D., Krueger, A., von Boehmer, H., Khazaie, K., and Gounari, F. (2006). c-Myc mediates pre-TCR-induced proliferation but not developmental progression. Blood *108*, 2669-2677.

Douagi, I., Vieira, P., and Cumano, A. (2002). Lymphocyte commitment during embryonic development, in the mouse. Semin Immunol *14*, 361-369.

Douglas, N. C., Jacobs, H., Bothwell, A. L., and Hayday, A. C. (2001). Defining the specific physiological requirements for c-Myc in T cell development. Nat Immunol *2*, 307-315.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol *10*, 262-267.

Dudley, E. C., Girardi, M., Owen, M. J., and Hayday, A. C. (1995). Alpha beta and gamma delta T cells can share a late common precursor. Curr Biol *5*, 659-669.

Eischen, C. M., Packham, G., Nip, J., Fee, B. E., Hiebert, S. W., Zambetti, G. P., and Cleveland, J. L. (2001a). Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. Oncogene *20*, 6983-6993.

Eischen, C. M., Woo, D., Roussel, M. F., and Cleveland, J. L. (2001b). Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol Cell Biol *21*, 5063-5070.

Engel, I., and Murre, C. (2004). E2A proteins enforce a proliferation checkpoint in developing thymocytes. Embo J *23*, 202-211.

Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G. B., Kobayashi, R., Hunter, T., and Lu, Z. (2007). Phosphorylation of beta -catenin by AKT promotes beta -catenin transcriptional activity. J Biol Chem.

Fayard, E., Tintignac, L. A., Baudry, A., and Hemmings, B. A. (2005). Protein kinase B/Akt at a glance. J Cell Sci *118*, 5675-5678.

Fehling, H. J., Iritani, B. M., Krotkova, A., Forbush, K. A., Laplace, C., Perlmutter, R. M., and von Boehmer, H. (1997). Restoration of thymopoiesis in pT alpha-/- mice by anti-CD3epsilon antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. Immunity *6*, 703-714.

Fehling, H. J., Krotkova, A., Saint-Ruf, C., and von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. Nature *375*, 795-798.

Felsher, D. W., and Bishop, J. M. (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell *4*, 199-207.

Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev *17*, 1115-1129.

Ferrando, A. A., Neuberg, D. S., Staunton, J., Loh, M. L., Huard, C., Raimondi, S. C., Behm, F. G., Pui, C. H., Downing, J. R., Gilliland, D. G., et al. (2002). Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell *1*, 75-87.

Forsberg, E. C., Serwold, T., Kogan, S., Weissman, I. L., and Passegue, E. (2006). New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. Cell *126*, 415-426.

Fruman, D. A. (2004). Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. Curr Opin Immunol *16*, 314-320.

Fukumoto, S., Hsieh, C. M., Maemura, K., Layne, M. D., Yet, S. F., Lee, K. H., Matsui, T., Rosenzweig, A., Taylor, W. G., Rubin, J. S., et al. (2001). Akt participation in the Wnt signaling pathway through Dishevelled. J Biol Chem *276*, 17479-17483.

Galandrini, R., Henning, S. W., and Cantrell, D. A. (1997). Different functions of the GTPase Rho in prothymocytes and late pre-T cells. Immunity *7*, 163-174.

Garbe, A. I., Krueger, A., Gounari, F., Zuniga-Pflucker, J. C., and von Boehmer, H. (2006). Differential synergy of Notch and T cell receptor signaling determines alphabeta versus gammadelta lineage fate. J Exp Med *203*, 1579-1590.

Gartel, A. L., Ye, X., Goufman, E., Shianov, P., Hay, N., Najmabadi, F., and Tyner, A. L. (2001). Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A *98*, 4510-4515.

Georgopoulos, K., Bigby, M., Wang, J. H., Molnar, A., Wu, P., Winandy, S., and Sharpe, A. (1994). The Ikaros gene is required for the development of all lymphoid lineages. Cell *79*, 143-156.

Godfrey, D. I., Kennedy, J., Mombaerts, P., Tonegawa, S., and Zlotnik, A. (1994). Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. J Immunol *152*, 4783-4792.

Godfrey, D. I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol *150*, 4244-4252.

Gomez, M., Tybulewicz, V., and Cantrell, D. A. (2000). Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. Nat Immunol *1*, 348-352.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., and Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev *15*, 1406-1418.

Gounari, F., Aifantis, I., Khazaie, K., Hoeflinger, S., Harada, N., Taketo, M. M., and von Boehmer, H. (2001). Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat Immunol *2*, 863-869.

Gounari, F., Aifantis, I., Martin, C., Fehling, H. J., Hoeflinger, S., Leder, P., von Boehmer, H., and Reizis, B. (2002). Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene. Nat Immunol *3*, 489-496.

Gounari, F., Chang, R., Cowan, J., Guo, Z., Dose, M., Gounaris, E., and Khazaie, K. (2005). Loss of adenomatous polyposis coli gene function disrupts thymic development. Nat Immunol *6*, 800-809.

Grandori, C., Gomez-Roman, N., Felton-Edkins, Z. A., Ngouenet, C., Galloway, D. A., Eisenman, R. N., and White, R. J. (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol *7*, 311-318.

Grewal, S. S., Li, L., Orian, A., Eisenman, R. N., and Edgar, B. A. (2005). Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nat Cell Biol *7*, 295-302.

Groettrup, M., Baron, A., Griffiths, G., Palacios, R., and von Boehmer, H. (1992). T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. Embo J *11*, 2735-2745.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M. J., Hayday, A. C., and von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. Cell *75*, 283-294.

Groves, T., Smiley, P., Cooke, M. P., Forbush, K., Perlmutter, R. M., and Guidos, C. J. (1996). Fyn can partially substitute for Lck in T lymphocyte development. Immunity *5*, 417-428.

Guo, Z., Dose, M., Kovalovsky, D., Chang, R., O'Neil, J., Look, A. T., von Boehmer, H., Khazaie, K., and Gounari, F. (2007). {beta}-Catenin stabilization stalls the transition from Double-

Positive to Single Positive stage and predisposes thymocytes to malignant transformation. Blood.

Hagenbeek, T. J., Naspetti, M., Malergue, F., Garcon, F., Nunes, J. A., Cleutjens, K. B., Trapman, J., Krimpenfort, P., and Spits, H. (2004). The loss of PTEN allows TCR alphabeta lineage thymocytes to bypass IL-7 and Pre-TCR-mediated signaling. J Exp Med *200*, 883-894.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. Embo J *18*, 5931-5942.

Harman, B. C., Jenkinson, W. E., Parnell, S. M., Rossi, S. W., Jenkinson, E. J., and Anderson, G. (2005). T/B lineage choice occurs prior to intrathymic Notch signaling. Blood *106*, 886-892.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

He, X. C., Yin, T., Grindley, J. C., Tian, Q., Sato, T., Tao, W. A., Dirisina, R., Porter-Westpfahl, K. S., Hembree, M., Johnson, T., et al. (2007). PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat Genet *39*, 189-198.

Hinton, H. J., Alessi, D. R., and Cantrell, D. A. (2004). The serine kinase phosphoinositide-dependent kinase 1 (PDK1) regulates T cell development. Nat Immunol *5*, 539-545.

Hoffman, B., Amanullah, A., Shafarenko, M., and Liebermann, D. A. (2002). The proto-oncogene c-myc in hematopoietic development and leukemogenesis. Oncogene *21*, 3414-3421.

Igarashi, H., Gregory, S. C., Yokota, T., Sakaguchi, N., and Kincade, P. W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity *17*, 117-130.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. Embo J *17*, 1371-1384.

Ioannidis, V., Beermann, F., Clevers, H., and Held, W. (2001). The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. Nat Immunol *2*, 691-697.

Iritani, B. M., Delrow, J., Grandori, C., Gomez, I., Klacking, M., Carlos, L. S., and Eisenman, R. N. (2002). Modulation of T-lymphocyte development, growth and cell size by the Myc antagonist and transcriptional repressor Mad1. Embo J *21*, 4820-4830.

Iritani, B. M., and Eisenman, R. N. (1999). c-Myc enhances protein synthesis and cell size during B lymphocyte development. Proc Natl Acad Sci U S A *96*, 13180-13185.

Itoh, K., Antipova, A., Ratcliffe, M. J., and Sokol, S. (2000). Interaction of dishevelled and Xenopus axin-related protein is required for wnt signal transduction. Mol Cell Biol *20*, 2228-2238.

Jacobs, H., Krimpenfort, P., Haks, M., Allen, J., Blom, B., Demolliere, C., Kruisbeek, A., Spits, H., and Berns, A. (1999). PIM1 reconstitutes thymus cellularity in interleukin 7- and common gamma chain-mutant mice and permits thymocyte maturation in Rag- but not CD3gamma-deficient mice. J Exp Med *190*, 1059-1068.

Jacobs, H., Vandeputte, D., Tolkamp, L., de Vries, E., Borst, J., and Berns, A. (1994). CD3 components at the surface of pro-T cells can mediate pre-T cell development in vivo. Eur J Immunol *24*, 934-939.

Jeffries, S., Robbins, D. J., and Capobianco, A. J. (2002). Characterization of a high-molecular-weight Notch complex in the nucleus of Notch(ic)-transformed RKE cells and in a human T-cell leukemia cell line. Mol Cell Biol *22*, 3927-3941.

Jiang, Q., Huang, J., Li, W. Q., Cavinato, T., Keller, J. R., and Durum, S. K. (2007). Role of the intracellular domain of IL-7 receptor in T cell development. J Immunol *178*, 228-234.

Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P., Roller, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000). The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J Biol Chem *275*, 16602-16608.

Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N., and Gallant, P. (1999). Drosophila myc regulates cellular growth during development. Cell *98*, 779-790.

Jonkers, J., Korswagen, H. C., Acton, D., Breuer, M., and Berns, A. (1997). Activation of a novel proto-oncogene, Frat1, contributes to progression of mouse T-cell lymphomas. Embo J *16*, 441-450.

Kanazawa, S., Ilic, D., Hashiyama, M., Okada, M., Noumura, T., Aizawa, S., and Suda, T. (1996). Impaired development of CD4+ CD8+ thymoyctes by csk-'knock-in' into fyn locus. Oncogene *13*, 199-204.

Kang, J., Volkmann, A., and Raulet, D. H. (2001). Evidence that gammadelta versus alphabeta T cell fate determination is initiated independently of T cell receptor signaling. J Exp Med *193*, 689-698.

Katoh, M. (2006). NUMB is a break of WNT-Notch signaling cycle. Int J Mol Med *18*, 517-521.

Kawamoto, H., Ikawa, T., Ohmura, K., Fujimoto, S., and Katsura, Y. (2000). T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. Immunity *12*, 441-450.

Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol *7*, 1048-1056.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M., and Kikuchi, A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol Cell Biol *19*, 4414-4422.

Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem *273*, 10823-10826.

Klemsz, M. J., Justement, L. B., Palmer, E., and Cambier, J. C. (1989). Induction of c-fos and c-myc expression during B cell activation by IL-4 and immunoglobulin binding ligands. J Immunol *143*, 1032-1039.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Konrad, M. A., and Zuniga-Pflucker, J. C. (2005). The BTG/TOB family protein TIS21 regulates stage-specific proliferation of developing thymocytes. Eur J Immunol *35*, 3030-3042.

Krueger, A., and von Boehmer, H. (2007). Identification of a T lineage-committed progenitor in adult blood. Immunity *26*, 105-116.

Labrecque, N., Whitfield, L. S., Obst, R., Waltzinger, C., Benoist, C., and Mathis, D. (2001). How much TCR does a T cell need? Immunity *15*, 71-82.

Lai, E. C. (2002). Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. EMBO Rep *3*, 840-845.

Law, L. W., Dunn, T. B., Trainin, N., and Levey, R. H. (1964). Studies of thymic function. Wistar Inst Symp Monogr *2*, 105-120.

Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Perez-Melgosa, M., Sweetser, M. T., Schlissel, M. S., Nguyen, S., et al. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity *15*, 763-774.

Lehar, S. M., Dooley, J., Farr, A. G., and Bevan, M. J. (2005). Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. Blood *105*, 1440-1447.

Levens, D. L. (2003). Reconstructing MYC. Genes Dev *17*, 1071-1077.

Lind, E. F., Prockop, S. E., Porritt, H. E., and Petrie, H. T. (2001). Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. J Exp Med *194*, 127-134.

Lindsten, T., June, C. H., and Thompson, C. B. (1988). Transcription of T cell antigen receptor genes is induced by protein kinase C activation. J Immunol *141*, 1769-1774.

Liu, C., Saito, F., Liu, Z., Lei, Y., Uehara, S., Love, P., Lipp, M., Kondo, S., Manley, N., and Takahama, Y. (2006). Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. Blood *108*, 2531-2539.

Livak, F., Petrie, H. T., Crispe, I. N., and Schatz, D. G. (1995). In-frame TCR delta gene rearrangements play a critical role in the alpha beta/gamma delta T cell lineage decision. Immunity *2*, 617-627.

Logan, C. Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.

Maclean, K. H., Keller, U. B., Rodriguez-Galindo, C., Nilsson, J. A., and Cleveland, J. L. (2003). c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL. Mol Cell Biol *23*, 7256-7270.

Maillard, I., Tu, L., Sambandam, A., Yashiro-Ohtani, Y., Millholland, J., Keeshan, K., Shestova, O., Xu, L., Bhandoola, A., and Pear, W. S. (2006). The requirement for Notch signaling at the beta-selection checkpoint in vivo is absolute and independent of the pre-T cell receptor. J Exp Med *203*, 2239-2245.

Maillard, I., Weng, A. P., Carpenter, A. C., Rodriguez, C. G., Sai, H., Xu, L., Allman, D., Aster, J. C., and Pear, W. S. (2004). Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. Blood *104*, 1696-1702.

Malissen, M., Pereira, P., Gerber, D. J., Malissen, B., and DiSanto, J. P. (1997). The common cytokine receptor gamma chain controls survival of gamma/delta T cells. J Exp Med *186*, 1277-1285.

Malstrom, S., Tili, E., Kappes, D., Ceci, J. D., and Tsichlis, P. N. (2001). Tumor induction by an Lck-MyrAkt transgene is delayed by mechanisms controlling the size of the thymus. Proc Natl Acad Sci U S A *98*, 14967-14972.

Mao, C., Tili-Michaille, E. G., Dose, M., Haks, M. C., Bear, S. E., Maroulakou, I., Horie, K., Gaitanaris, G. A., Fidanza, V., Ludwig, T., et al. (2007). Unequal Contribution of Akt Isoforms in the Double-Negative to Double-Positive Thymocyte Transition. J Immunol, in press.

Marinkovic, D., Marinkovic, T., Mahr, B., Hess, J., and Wirth, T. (2004). Reversible lymphomagenesis in conditionally c-MYC expressing mice. Int J Cancer *110*, 336-342.

Martin, C. H., Aifantis, I., Scimone, M. L., von Andrian, U. H., Reizis, B., von Boehmer, H., and Gounari, F. (2003). Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. Nat Immunol *4*, 866-873.

Masuda, K., Kubagawa, H., Ikawa, T., Chen, C. C., Kakugawa, K., Hattori, M., Kageyama, R., Cooper, M. D., Minato, N., Katsura, Y., and Kawamoto, H. (2005). Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. Embo J *24*, 4052-4060.

Mateyak, M. K., Obaya, A. J., Adachi, S., and Sedivy, J. M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ *8*, 1039-1048.

Matsuzaki, Y., Gyotoku, J., Ogawa, M., Nishikawa, S., Katsura, Y., Gachelin, G., and Nakauchi, H. (1993). Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. J Exp Med *178*, 1283-1292.

Melichar, H. J., Narayan, K., Der, S. D., Hiraoka, Y., Gardiol, N., Jeannet, G., Held, W., Chambers, C. A., and Kang, J. (2007). Regulation of gammadelta versus alphabeta T lymphocyte differentiation by the transcription factor SOX13. Science *315*, 230-233.

Miller, J. F. (1961). Immunological function of the thymus. Lancet *2*, 748-749.

Miller, J. F. (2002). The discovery of thymus function and of thymus-derived lymphocytes. Immunol Rev *185*, 7-14.

Miller, J. R. (1962). Effect of cortisone on the developing mouse foetus. Nature *194*, 891-892.

Misslitz, A., Bernhardt, G., and Forster, R. (2006). Trafficking on serpentines: molecular insight on how maturating T cells find their winding paths in the thymus. Immunol Rev *209*, 115-128.

Misslitz, A., Pabst, O., Hintzen, G., Ohl, L., Kremmer, E., Petrie, H. T., and Forster, R. (2004). Thymic T cell development and progenitor localization depend on CCR7. J Exp Med *200*, 481-491.

Mitchell, K. O., and El-Deiry, W. S. (1999). Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. Cell Growth Differ *10*, 223-230.

Miyazaki, T., Liu, Z. J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Permutter, R. M., and Taniguchi, T. (1995). Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell *81*, 223-231.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., and et al. (1992). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. Nature *360*, 225-231.

Mori, S., Shortman, K., and Wu, L. (2001). Characterization of thymus-seeding precursor cells from mouse bone marrow. Blood *98*, 696-704.

Mukherjee, S., and Conrad, S. E. (2005). c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem *280*, 17617-17625.

Nicholson, K. M., and Anderson, N. G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal *14*, 381-395.

Nilsson, J. A., and Cleveland, J. L. (2003). Myc pathways provoking cell suicide and cancer. Oncogene *22*, 9007-9021.

Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse, R. (1994). dishevelled and armadillo act in the wingless signalling pathway in Drosophila. Nature *367*, 80-83.

Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795-801.

Obaya, A. J., Mateyak, M. K., and Sedivy, J. M. (1999). Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene *18*, 2934-2941.

Okamura, R. M., Sigvardsson, M., Galceran, J., Verbeek, S., Clevers, H., and Grosschedl, R. (1998). Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. Immunity *8*, 11-20.

Outram, S. V., Varas, A., Pepicelli, C. V., and Crompton, T. (2000). Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. Immunity *13*, 187-197.

Palomero, T., Lim, W. K., Odom, D. T., Sulis, M. L., Real, P. J., Margolin, A., Barnes, K. C., O'Neil, J., Neuberg, D., Weng, A. P., et al. (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A *103*, 18261-18266.

Pear, W. S., Aster, J. C., Scott, M. L., Hasserjian, R. P., Soffer, B., Sklar, J., and Baltimore, D. (1996). Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med *183*, 2283-2291.

Pearse, M., Wu, L., Egerton, M., Wilson, A., Shortman, K., and Scollay, R. (1989). A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. Proc Natl Acad Sci U S A *86*, 1614-1618.

Pearson, R., and Weston, K. (2000). c-Myb regulates the proliferation of immature thymocytes following beta-selection. Embo J *19*, 6112-6120.

Pelengaris, S., and Khan, M. (2003). The many faces of c-MYC. Arch Biochem Biophys *416*, 129-136.

Perry, S. S., Wang, H., Pierce, L. J., Yang, A. M., Tsai, S., and Spangrude, G. J. (2004). L-selectin defines a bone marrow analog to the thymic early T-lineage progenitor. Blood *103*, 2990-2996.

Perumal, N. B., Kenniston, T. W., Jr., Tweardy, D. J., Dyer, K. F., Hoffman, R., Peschon, J., and Appasamy, P. M. (1997). TCR-gamma genes are rearranged but not transcribed in IL-7R alpha-deficient mice. J Immunol *158*, 5744-5750.

Petrie, H. T. (2003). Cell migration and the control of post-natal T-cell lymphopoiesis in the thymus. Nat Rev Immunol *3*, 859-866.

Pivniouk, V. I., and Geha, R. S. (2000). The role of SLP-76 and LAT in lymphocyte development. Curr Opin Immunol *12*, 173-178.

Plotkin, J., Prockop, S. E., Lepique, A., and Petrie, H. T. (2003). Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. J Immunol *171*, 4521-4527.

Pongracz, J. E., Parnell, S. M., Jones, T., Anderson, G., and Jenkinson, E. J. (2006). Overexpression of ICAT highlights a role for catenin-mediated canonical Wnt signalling in early T cell development. Eur J Immunol *36*, 2376-2383.

Porritt, H. E., Rumfelt, L. L., Tabrizifard, S., Schmitt, T. M., Zuniga-Pflucker, J. C., and Petrie, H. T. (2004). Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity *20*, 735-745.

Prockop, S. E., Palencia, S., Ryan, C. M., Gordon, K., Gray, D., and Petrie, H. T. (2002). Stromal cells provide the matrix for migration of early lymphoid progenitors through the thymic cortex. J Immunol *169*, 4354-4361.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity *10*, 547-558.

Rathmell, J. C., Fox, C. J., Plas, D. R., Hammerman, P. S., Cinalli, R. M., and Thompson, C. B. (2003). Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. Mol Cell Biol *23*, 7315-7328.

Reif, K., Nobes, C. D., Thomas, G., Hall, A., and Cantrell, D. A. (1996). Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. Curr Biol *6*, 1445-1455.

Reisman, D., Elkind, N. B., Roy, B., Beamon, J., and Rotter, V. (1993). c-Myc trans-activates the p53 promoter through a required downstream CACGTG motif. Cell Growth Differ *4*, 57-65.

Reizis, B., and Leder, P. (2002). Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. Genes Dev *16*, 295-300.

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Rodewald, H. R., and Fehling, H. J. (1998). Molecular and cellular events in early thymocyte development. Adv Immunol *69*, 1-112.

Rodewald, H. R., Kretzschmar, K., Takeda, S., Hohl, C., and Dessing, M. (1994). Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. Embo J *13*, 4229-4240.

Rodriguez-Borlado, L., Barber, D. F., Hernandez, C., Rodriguez-Marcos, M. A., Sanchez, A., Hirsch, E., Wymann, M., Martinez, A. C., and Carrera, A. C. (2003). Phosphatidylinositol 3-kinase regulates the CD4/CD8 T cell differentiation ratio. J Immunol *170*, 4475-4482.

Rossi, F. M., Corbel, S. Y., Merzaban, J. S., Carlow, D. A., Gossens, K., Duenas, J., So, L., Yi, L., and Ziltener, H. J. (2005). Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nat Immunol *6*, 626-634.

Saint-Ruf, C., Panigada, M., Azogui, O., Debey, P., von Boehmer, H., and Grassi, F. (2000). Different initiation of pre-TCR and gammadeltaTCR signalling. Nature *406*, 524-527.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J., and von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. Science *266*, 1208-1212.

Salvador, J. M., Hollander, M. C., Nguyen, A. T., Kopp, J. B., Barisoni, L., Moore, J. K., Ashwell, J. D., and Fornace, A. J., Jr. (2002). Mice lacking the p53-effector gene Gadd45a develop a lupus-like syndrome. Immunity *16*, 499-508.

Sambandam, A., Maillard, I., Zediak, V. P., Xu, L., Gerstein, R. M., Aster, J. C., Pear, W. S., and Bhandoola, A. (2005). Notch signaling controls the generation and differentiation of early T lineage progenitors. Nat Immunol *6*, 663-670.

Sansom, O. J., Meniel, V. S., Muncan, V., Phesse, T. J., Wilkins, J. A., Reed, K. R., Vass, J. K., Athineos, D., Clevers, H., and Clarke, A. R. (2007). Myc deletion rescues Apc deficiency in the small intestine. Nature.

Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., et al. (2000). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science *287*, 1040-1046.

Satoh, Y., Matsumura, I., Tanaka, H., Ezoe, S., Sugahara, H., Mizuki, M., Shibayama, H., Ishiko, E., Ishiko, J., Nakajima, K., and Kanakura, Y. (2004). Roles for c-Myc in self-renewal of hematopoietic stem cells. J Biol Chem *279*, 24986-24993.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol *7*, 1037-1047.

Schilham, M. W., Wilson, A., Moerer, P., Benaissa-Trouw, B. J., Cumano, A., and Clevers, H. C. (1998). Critical involvement of Tcf-1 in expansion of thymocytes. J Immunol *161*, 3984-3991.

Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S., and Zuniga-Pflucker, J. C. (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol *5*, 410-417.

Schmitt, T. M., and Zuniga-Pflucker, J. C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity *17*, 749-756.

Schuhmacher, M., Staege, M. S., Pajic, A., Polack, A., Weidle, U. H., Bornkamm, G. W., Eick, D., and Kohlhuber, F. (1999). Control of cell growth by c-Myc in the absence of cell division. Curr Biol *9*, 1255-1258.

Schwarz, B. A., Sambandam, A., Maillard, I., Harman, B. C., Love, P. E., and Bhandoola, A. (2007). Selective thymus settling regulated by cytokine and chemokine receptors. J Immunol *178*, 2008-2017.

Scimone, M. L., Aifantis, I., Apostolou, I., von Boehmer, H., and von Andrian, U. H. (2006). A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. Proc Natl Acad Sci U S A *103*, 7006-7011.

Seminario, M. C., and Wange, R. L. (2003). Lipid phosphatases in the regulation of T cell activation: living up to their PTEN-tial. Immunol Rev *192*, 80-97.

Shinkai, Y., and Alt, F. W. (1994). CD3 epsilon-mediated signals rescue the development of CD4+CD8+ thymocytes in RAG-2-/- mice in the absence of TCR beta chain expression. Int Immunol *6*, 995-1001.

Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A. A., Levin, S. D., Geng, Y., von Boehmer, H., and Sicinski, P. (2003). Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell *4*, 451-461.

Sitnicka, E., Bryder, D., Theilgaard-Monch, K., Buza-Vidas, N., Adolfsson, J., and Jacobsen, S. E. (2002). Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. Immunity *17*, 463-472.

Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999). Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. Embo J *18*, 2823-2835.

Spanopoulou, E., Early, A., Elliott, J., Crispe, N., Ladyman, H., Ritter, M., Watt, S., Grosveld, F., and Kioussis, D. (1989). Complex lymphoid and epithelial thymic tumours in Thy1-myc transgenic mice. Nature *342*, 185-189.

Staal, F. J., and Clevers, H. C. (2003). Wnt signaling in the thymus. Curr Opin Immunol *15*, 204-208.

Staal, F. J., Meeldijk, J., Moerer, P., Jay, P., van de Weerdt, B. C., Vainio, S., Nolan, G. P., and Clevers, H. (2001). Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. Eur J Immunol *31*, 285-293.

Taghon, T., Yui, M. A., Pant, R., Diamond, R. A., and Rothenberg, E. V. (2006). Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity *24*, 53-64.

Tanigaki, K., Tsuji, M., Yamamoto, N., Han, H., Tsukada, J., Inoue, H., Kubo, M., and Honjo, T. (2004). Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. Immunity *20*, 611-622.

Tavtigian, S. V., Zabludoff, S. D., and Wold, B. J. (1994). Cloning of mid-G1 serum response genes and identification of a subset regulated by conditional myc expression. Mol Biol Cell *5*, 375-388.

Terszowski, G., Muller, S. M., Bleul, C. C., Blum, C., Schirmbeck, R., Reimann, J., Pasquier, L. D., Amagai, T., Boehm, T., and Rodewald, H. R. (2006). Evidence for a functional second thymus in mice. Science *312*, 284-287.

Trumpp, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G. R., and Bishop, J. M. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature *414*, 768-773.

Uehara, S., Grinberg, A., Farber, J. M., and Love, P. E. (2002). A role for CCR9 in T lymphocyte development and migration. J Immunol *168*, 2811-2819.

Vaillant, F., Blyth, K., Andrew, L., Neil, J. C., and Cameron, E. R. (2002). Enforced expression of Runx2 perturbs T cell development at a stage coincident with beta-selection. J Immunol *169*, 2866-2874.

van de Wetering, M., de Lau, W., and Clevers, H. (2002a). WNT signaling and lymphocyte development. Cell *109 Suppl*, S13-19.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P., et al. (2002b). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.

van Lohuizen, M., Verbeek, S., Scheijen, B., Wientjens, E., van der Gulden, H., and Berns, A. (1991). Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. Cell *65*, 737-752.

von Boehmer, H. (1997). Aspects of lymphocyte developmental biology. Immunol Today *18*, 260-262.

von Boehmer, H., and Fehling, H. J. (1997). Structure and function of the pre-T cell receptor. Annu Rev Immunol *15*, 433-452.

von Freeden-Jeffry, U., Solvason, N., Howard, M., and Murray, R. (1997). The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity *7*, 147-154.

Walker, W., Zhou, Z. Q., Ota, S., Wynshaw-Boris, A., and Hurlin, P. J. (2005). Mnt-Max to Myc-Max complex switching regulates cell cycle entry. J Cell Biol *169*, 405-413.

Wallis, V. J., Leuchars, E., Chwalinski, S., and Davies, A. J. (1975). On the sparse seeding of bone marrow and thymus in radiation chimaeras. Transplantation *19*, 2-11.

Wange, R. L., Guitian, R., Isakov, N., Watts, J. D., Aebersold, R., and Samelson, L. E. (1995). Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. J Biol Chem *270*, 18730-18733.

Wange, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993). Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor zeta and CD3 epsilon from activated Jurkat T cells. J Biol Chem *268*, 19797-19801.

Waters, C. M., Littlewood, T. D., Hancock, D. C., Moore, J. P., and Evan, G. I. (1991). c-myc protein expression in untransformed fibroblasts. Oncogene *6*, 797-805.

Weerkamp, F., Baert, M. R., Naber, B. A., Koster, E. E., de Haas, E. F., Atkuri, K. R., van Dongen, J. J., Herzenberg, L. A., and Staal, F. J. (2006a). Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules. Proc Natl Acad Sci U S A *103*, 3322-3326.

Weerkamp, F., Luis, T. C., Naber, B. A., Koster, E. E., Jeannotte, L., van Dongen, J. J., and Staal, F. J. (2006b). Identification of Notch target genes in uncommitted T-cell progenitors: No direct induction of a T-cell specific gene program. Leukemia *20*, 1967-1977.

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., and Aster, J. C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science *306*, 269-271.

Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C. G., Sai, H., Tobias, J., et al. (2006). c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev *20*, 2096-2109.

Wiest, D. L., Burgess, W. H., McKean, D., Kearse, K. P., and Singer, A. (1995). The molecular chaperone calnexin is expressed on the surface of immature thymocytes in association with clonotype-independent CD3 complexes. Embo J *14*, 3425-3433.

Wilson, A., MacDonald, H. R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med *194*, 1003-1012.

Wilson, A., Murphy, M. J., Oskarsson, T., Kaloulis, K., Bettess, M. D., Oser, G. M., Pasche, A. C., Knabenhans, C., Macdonald, H. R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Winandy, S., Wu, L., Wang, J. H., and Georgopoulos, K. (1999). Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. J Exp Med *190*, 1039-1048.

Wolfer, A., Bakker, T., Wilson, A., Nicolas, M., Ioannidis, V., Littman, D. R., Lee, P. P., Wilson, C. B., Held, W., MacDonald, H. R., and Radtke, F. (2001). Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. Nat Immunol *2*, 235-241.

Wolfer, A., Wilson, A., Nemir, M., MacDonald, H. R., and Radtke, F. (2002). Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. Immunity *16*, 869-879.

Wu, L., Antica, M., Johnson, G. R., Scollay, R., and Shortman, K. (1991). Developmental potential of the earliest precursor cells from the adult mouse thymus. J Exp Med *174*, 1617-1627.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet *26*, 484-489.

Xu, Y., Banerjee, D., Huelsken, J., Birchmeier, W., and Sen, J. M. (2003). Deletion of beta-catenin impairs T cell development. Nat Immunol *4*, 1177-1182.

Yamasaki, S., Ishikawa, E., Sakuma, M., Ogata, K., Sakata-Sogawa, K., Hiroshima, M., Wiest, D. L., Tokunaga, M., and Saito, T. (2006). Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. Nat Immunol *7*, 67-75.

Yoshida, T., Ng, S. Y., Zuniga-Pflucker, J. C., and Georgopoulos, K. (2006). Early hematopoietic lineage restrictions directed by Ikaros. Nat Immunol *7*, 382-391.

Zeller, K. I., Jegga, A. G., Aronow, B. J., O'Donnell, K. A., and Dang, C. V. (2003). An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. Genome Biol *4*, R69.

Zhang, X., Ma, L., Enkemann, S. A., and Pledger, W. J. (2003). Role of Gadd45alpha in the density-dependent G1 arrest induced by p27(Kip1). Oncogene *22*, 4166-4174.

Zweidler-McKay, P. A., and Pear, W. S. (2004). Notch and T cell malignancy. Semin Cancer Biol *14*, 329-340.

8 APPENDIX

8.1 Abbreviations

7-AAD	7- Amino Actinomycin D
aa	amino acid
α-CD3ε	anti-CD3 epsilon antibody
Akt	thymoma viral proto-oncogene 1
APC	Allophycocyanin
Apc	Adenomatous polyposis coli
B220	protein tyrosine phosphatase, receptor type, C
bp	base pair
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
c-Fyn	Fyn proto-oncogene
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CMV	Cytomegalovirus
c-Myc	cellular myelocytomatosis oncogene
Csk	C-terminal Src kinase
Ctnnb	beta-catenin
CTP	circulating T cell progenitor
Cy	Cyanide dye
DC	Dendritic cell
DL	Delta-like
DMSO	Dimethyl sulfoxide
DN	double-negative
DN	dominant negative
DP	double-positive
Dsv	Dishevelled
E	Embryonic day
E2A	E2A immunoglobulin enhancer binding factors E12/E47

ECL	enhanced chemiluminescence
EGF	Epidermal growth factor
ELP	early lymphocyte progenitor
ES	embryonic stem
ETP	early T lineage progenitor
FACS	Fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
FOXO	forkhead box, sub-group O
FRAT1	frequently rearranged in advanced T-cell lymphomas
Fzd	Frizzled
$\gamma\delta$ T cell	T cell receptor gamma and delta positive cell
GFP	Green fluorescent protein
HA	hemagglutinin
Hes	hairy enhancer of split
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
i.p.	intraperitoneal
IC	intracellular
ICAT	inhibitor of beta-catenin and Tcf-4
IFN	Interferon
Ig	Immunoglobulin
IκB	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta
IL7	Interleukin 7
IL7-R	Interleukin 7 receptor
ISP	immature single-positive
LAT	linker for activation of T cells
Lck	lymphocyte protein tyrosine kinase
Lef	lymphocyte enhancer factor
lin^-	lineage marker negative

LSK	Lin- Sca1+ c-kit+
MAML	Mastermind-like
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumor virus
MPP	multipotent progenitor
MyrAkt	myristoylated Akt
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor of kappa light chain gene enhancer in B-cells
NK	Natural killer
o/n	over night
OD	Optical density
PCR	Polymerase chain reaction
PE	Phyco-erythrin
PerCP	peridinin chlorophyll protein
PI(3)	phosphatidylinositol-3
Pim1	proviral integration site 1
preTCR	pre-T cell receptor
pTα	pre-T cell receptor alpha
RAG	Recombinase activating gene
rpm	revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcription dependent polymerase chain reaction
Runx2	runt related transcription factor 2
Sca1	lymphocyte antigen 6 complex, locus A
Scid	Severe combined immunodeficiency
SCZ	subcapsular zone
SP	single-positive
T-ALL	T-cell acute lymphoplastic leukemia
Tcf	T cell factor

TCR	T cell receptor
TCRα	T cell receptor alpha
TCRβ	T cell receptor beta
TEC	thymic epithelial cell
TGF	Transforming growth factor
Thy-1	thymus cell antigen 1
TSP	thymus-seeding progenitor
Wnt	wingless-related MMTV integration site 1
wt	wildtype
Zap-70	zeta-chain (TCR) associated protein kinase 70kDa

8.2 Publications

Publications related to this work:

Dose M, Khan I, Guo Z, Kovalovsky D, Krueger A, von Boehmer H, Khazaie K, Gounari F
"c-Myc mediates pre-TCR-induced proliferation but not developmental progression"
Blood. 2006 Oct 15;108(8):2669-77

Mao C, Tili-Michaille EG, Dose M, Haks MC, Bear SE, Maroulakou I, Horie K, Gaitanaris GA, Fidanza V, Ludwig T, Wiest DL, Gounari F, and Tsichlis PN
"Unequal Contribution of Akt Isoforms in the Double-Negative to Double-Positive Thymocyte Transition"
J Immunol 2007, *in press*

Dose M and Gounari F
"Wnt and c-Myc signaling at the preTCR checkpoint", poster presentation at the Keystone Symposium 2007 "Stem Cell Interactions with their Microenvironemtal Niche", Keystone, CO

Dose M and Gounari F
"c-Myc mediates preTCR induced proliferation but not developmental progression", poster presentation at the American Association of Immunologists (AAI) meeting 2006, Boston, MA

Dose M, Khan I, and Gounari F
"c-Myc is required for the preTCR induced proliferation wave but does not affect differentiation or apoptosis", prize-winning poster at the Immunology Program Retreat 2005, Tufts University Sackler School of Graduate Biomedical Sciences, Boston, MA

Additional publications:

Guo Z, Dose M, Kovalovsky D, Chang R, O'Neil J, Look AT, von Boehmer H, Khazaie K, Gounari F
"β-Catenin stabilization stalls the transition from Double-Positive to Single Positive stage and predisposes thymocytes to malignant transformation"
Blood. 2007 Feb 22; [Epub ahead of print]

Gounari F, Chang R, Cowan J, Guo Z, Dose M, Gounaris E, Khazaie K.
"Loss of adenomatous polyposis coli gene function disrupts thymic development"
Nat Immunol. 2005 Aug;6(8):800

8.3 Curriculum Vitae

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Diplomarbeit:

18.02.03 – 08.08.03	“Characterization of RanBPM2, a novel β1 tubulin binding protein in primary murine megakaryocytes” am Dana-Farber Cancer Institute, Boston, unter Anleitung von Prof. Ramesh Shivdasani, M.D., PhD

Studium:

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Hochschulzugangsberechtigung:

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Derzeitige Beschäftigung:

seit dem 01.11.03	Wissenschaftliche Tätigkeit am Tufts- New England Medical Center (NEMC) in Boston, MA, am Molecular Oncology Research Institute (MORI) in dem Labor von Prof. Fotini Gounari, PhD, H.D.R.

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