Canonical and non-canonical Wnt signaling in hematopoiesis and lymphocyte development

Colophon

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PhD thesis

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Cover: A balance between self-renewal and differentiation of hematopoietic stem cells (HSCs), is required to maintain the homeostasis of the hematopoietic system in order to combat against stress situations. **This balance** is partly regulated via the cross-talk of canonical and non-canonical Wnt signaling (like the balance of a traditional weight scale) to secure an optimal dosage required at different stages.

Canonical and non-canonical Wnt signaling in hematopoiesis and lymphocyte development

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To the dearest person in my life, Sepanta Famili

To my father and mother who made this possible

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CHAPTER 1

General Introduction

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Hematopoietic stem cells (HSCs) as source for all blood cells

The blood in our body consists of many different cell types. HSCs, which reside in the bone marrow (BM), are able to produce all the different cells present in our blood system, including platelets, red blood cells, and white blood cells. This involves a highly controlled process of both self-renewal, to maintain the pool of HSCs and differentiation. The processes of self-renewal and differentiation are coordinated by many signaling pathways, such as Notch [1], Wnt [2], BMP [3] and several others [4]. Aberrancies in genes constituting these pathways, either congenital or acquired, can influence these processes, eventually leading to arrests in development or to the development of hematological malignancies.

Under normal circumstances, HSCs give rise to all white blood cells, including both innate and adaptive immune cells. The innate immune system is already present at birth and is a nonspecific defense against pathogens and therefore is able to respond quickly. It is comprised of different cells types, including mast cells, macrophages, neutrophils, eosinophils, dendritic cells and natural killer (NK) cells. The cells of the adaptive immune system, comprised of B cells and T cells, are also present at birth similar to the cells from the innate immune system. However, cells from the adaptive immune system respond in an antigen-specific manner. These cells express receptors specific for antigens and upon antigen encounter, they will proliferate but also form memory cells. These memory cells are able to respond quicker upon a second encounter with the same antigen; a characteristic that is made use of by vaccination, thereby providing protection against the pathogen. The adaptive immune system is only found in vertebrates [5-7].

HSCs are rare cells that are difficult to characterize precisely by marker expression alone. The most robust criterion to determine true stem cell potential is the ability to provide long-term repopulation of an entire host with all hematopoietic lineages [8]. In mice, this is often assessed by performing transplantations into secondary recipients to determine self-renewal capacity [9, 10]. For human HSCs, this is of course not feasible in a clinical setting. Murine HSCs are characterized by the expression of Sca-1, C-kit, low expression levels of the Thy-1, low to absent expression of CD34 and the lack of lineage markers (e.g., B220, Mac-1, Gr-1, CD3, CD4, CD8 and Ter119). The most widely used HSC population in the mouse is the so called LSK population: lineage marker negative, Sca-1+, and C-kit+. Within this population, at least three subsets can be distinguished, namely, long-term [11-14] and short-term [12] HSCs, often by using CD34 in combination with the FLT3 marker and so-called Multipotent progenitors (MPP) that have largely lost true self-renewal capacity. Other markers are continuously evaluated and added in an attempt to more precisely define true HSCs. Of note are the so-called SLAM markers CD50 and CD48 which further subdivide the LSK population into cells enriched for long-term or short term repopulating stem cells and multipotent progenitors [15, 16].

Human HSCs and their clinical use

The regenerative capacity of HSC is of great use in the clinic for the treatment of many diseases affecting the blood system; leukemia, lymphoma, SCID, and hemoglobinopathies, encompassing thalassemia and sickle cell disease [17]. Either autologous or allogeneic stem cells are used for transplantation, often depending on the availability of donor material. As a first step in the transplantation procedure, the cells of the immune system in the patient are often depleted by chemotherapy, which is called conditioning, and then the patient will receive donor-derived HSC that can engraft and develop a new healthy immune system. HSCs can be isolated from different sources; BM, mobilized peripheral blood and umbilical cord blood, all of which are used in the clinic for transplantation [18]. In a clinical setting, the CD34+ fraction is used for transplantation as these cells can be isolated in a good laboratory practice (GLP) setting. However, already in 1997, it was described that the phenotype of HSCs could be further refined to CD34+CD38- containing a frequency of 1 in 617 cells with true HSC potential, defined by the capacity to repopulate a NOD/SCID mouse [19]. Thereafter, it was shown that this cell fraction can be divided into 3 groups based on the expression of both CD90 and CD45RA. The Lin-CD34+CD38- CD90+CD45RA- cell population isolated from umbilical cord blood was demonstrated to have multi-lineage BM engraftment potential when 10 cells were transplanted [20]. This cell population could be further subdivided by CD49f discrimination of which the CD49f+ population contained a frequency of LT-HSC of 1 in 10.5 cells [21]. This illustrates that currently, we are not yet able to identify the one cell phenotype that is most primitive and contains the highest long term repopulating capacity. Currently, the human HSC is described to be most enriched within the Lin-CD34+CD38-CD45RA-CD90+CD49f+ population followed by the MPP that has lost expression of both CD90 and CD49f [22] (Figure 1). From the MPP two cell types branch off; the CD34+CD38-CD45RA+CD90- MLP (multi-lymphoid progenitor) that can give rise to NK, B, and T cells, and the Lin- CD34+CD38+CD45RA-CD135+ CMP (common myeloid progenitor) that can give rise to the megakaryocytic-erythroid progenitor (MEP) and granulocyte-monocyte progenitor (GMP) [23]. The MLP is similar to common lymphoid progenitor (CLP), a progenitor proposed to be a precursor for lymphocytes but not for myeloid cells derived from many studies on hematopoiesis in the mouse [24-26]. In humans, this has been studied less extensively. The MLP comprises mostly lymphoid restricted cells, but also has some myeloid developmental potential, hence it cannot be considered as the human counterpart of the mouse common lymphoid progenitor. Instead, this population seems closer to lymphoid-primed multipotent progenitors, the so-called LMPPs [27]. Importantly, recent insights have identified a novel lineage of lymphocytes, the innate like lymphocytes (ILC) that do not express antigen-specific receptors but share many other properties with T cells [28-30]. Three subgroups are commonly distinguished based on the cytokines they produce and the transcription factors required for their development, a characteristic they also share with specific T cells

subsets. The classical NK cells are now referred to as part of the ILC1 cells and Lymphoid tissue inducer cells belong to the ILC3 family. Cells from the myeloid lineage, erythrocytes and granulocytes are progeny from the MEP and GMP progenitor types. Also on the gene expression level, there is a separation between lymphoid fate and a myeloid fate at the MLP stage [31]. Many of the transcription factors that are important in HSCs are known to be causative of leukemia when deregulated, for example, RUNX1, MLL, SCL/TAL1 and LMO2 [32, 33]. Under homeostatic conditions, the number of stem cells has to stay constant, which can be achieved by asymmetric cell division, through which one daughter cell keeps the stem cell identity and the other differentiates. The mechanism regulating asymmetric vs. symmetric remain poorly understood in mammals, particularly in HSCs. Depending on their localization, HSCS can divide symmetrically to expand the HSC pool (for instance during embryonic life in the fetal liver) or asymmetrically [34-36]. Adult HSCs are mostly quiescent. Indeed, most of the true stem cell activity is present in dormant LT-HSCs [37]. Additionally, the activation of dormant stem cells, for instance by inflammatory signals such as interferons, seems to be reversible as cycling HSCs return to the dormancy upon re-attainment of homeostasis [38]. Dormancy is thought to be a protective mechanism against exhaustion of HSCs, despite self-renewal properties, as there might be a limited self-renewal potential [39].

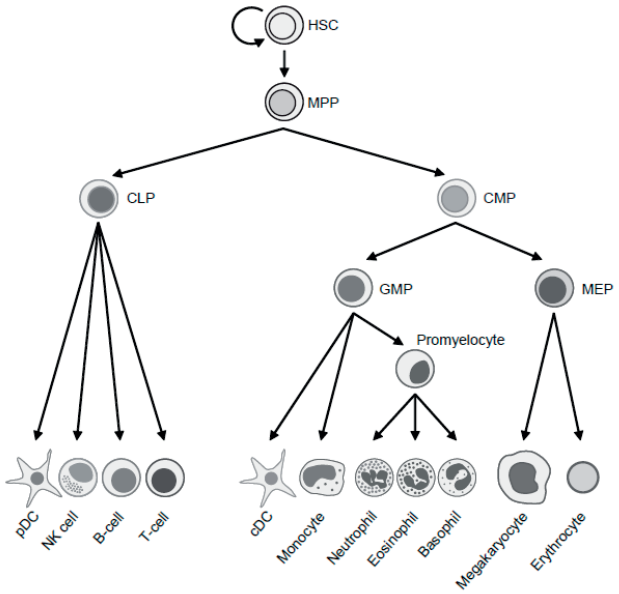

Figure 1. Schematic representation of the main lineage commitment steps in hematopoiesis.

HSCs with the self-renewal capacity are placed at the top of the hierarchy, develop to several multipotent progenitors which give rise to mature blood cells through a step wise process of lineage commitment. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-monocyte progenitor, MEP, megakaryocyte-monocyte progenitor, pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; NK, natural killer

Embryonic origin of HSCs

Blood tissues originate from mesoderm lineages during the embryogenesis. The first restricted cell types called hemangioblast which is thought to be derived from the common precursor of endothelial and hematopoietic lineages, [5, 32, 40]. However, it is difficult to determine the origin of the hematopoietic lineage due to the high mobility of the blood, and diverse localization of hematopoietic cells during embryogenesis, until the definitive hematopoietic organs are fully formed and functional.

Yolk sac (YS) is the first site of hematopoiesis in the mouse, which is formed between embryonic days 7 (E7) and E8 when the circulation is not established yet. This temporary hematopoietic organ mainly produces primitive erythrocytes cells and myeloid progenitors [41, 42]. Subsequently, adult HSCs are developed around E10.5 in the para-aortic splanchnopleura-(pSp-) also called aorta-gonads-mesonephros (AGM) region [43, 44]. These cells are colocalized with the endothelial cells in the ventral region of the dorsal aorta, [45], which raised the possibility of the development a "hemogenic endothelium" lineage as mentioned previously [46-49]. However, another hypothesis suggests that hematopoietic precursors are developed in sub aortic patches and then these cells migrate to the dorsal aorta via mesenchyme [50]. Immediately after being developed in the AGM region, HSCs can be found in other tissues such as the YS, placenta and fetal liver [51]. It is plausible that HSCs are developed in these organs de novo, or due to the local expansion. It has been proven that the hematopoietic cells migrate from the AGM, YS, and placenta into the fetal liver, which becomes the main hematopoietic organ until birth when BM hematopoiesis is established [52, 53]. Several studies have shown that human embryonic hematopoiesis follows very similar pattern to the murine fetal hematopoiesis [54] (Figure 2).

In the fetus, the liver is a major organ for HSC expansion and differentiation [55]. Although common precursors of myeloid and erythroid cells can be detected in the liver as early as E9.5, the first definitive HSCs seed the fetal liver at E11.5. The first phenomenon after colonization is a massive expansion of HSCs (38-fold between E12 and E16) which has been proven via competitive repopulating experiments. As HSCs start to emigrate from the liver into other organs such as the spleen and BM around E16, their repopulating potential decreases [56] (Figure 2). Despite adult HSCs, fetal HSCs are actively cycling, and they have greater self-renewal potential compared to the BM HSCs [9, 57].

The balance between HSC repopulation and differentiation toward mature blood cells is crucial to maintain enough HSC pool in one hand and to provide quick and sufficient blood cells during injury on the other hand. This balance is controlled by multiple evolutionary conserved signaling pathways including the Wnt, Notch, Smad, and Hedgehog pathways.

A large body of evidence has shown the presence of a complex molecular cross-talk between HSCs and the niche cells in their close vicinity, leading to the definition of one adhesion and signaling unit termed “stem-cell-niche synapse”, in analogy to the neuronal and immunological synapses [58]. In order to maintain the stem cell niche integrity, two different interactions are required. One to provide the adhesion among cells, and another to provide adhesion to the extracellular matrix, in order to trigger the activation of specific signaling pathways which potentially influence HSCs fate decisions, survival, and proliferation. The induction of signaling pathways can be mediated via three distinct mechanisms. Notch signaling is induced by cell-cell interactions via the binding of membrane associated ligands and receptors. Other signaling pathways such as Wnt, Smad/TGF/BMP/Activin, and Hedgehog are activated through binding of soluble factors to specific receptors located both on the HSCs and on the niche cell. Thirdly, hematopoietic cytokines like stem cell factor and thrombopoietin play an important role via binding to the Kit receptor, and the Mpl receptor respectively [58].

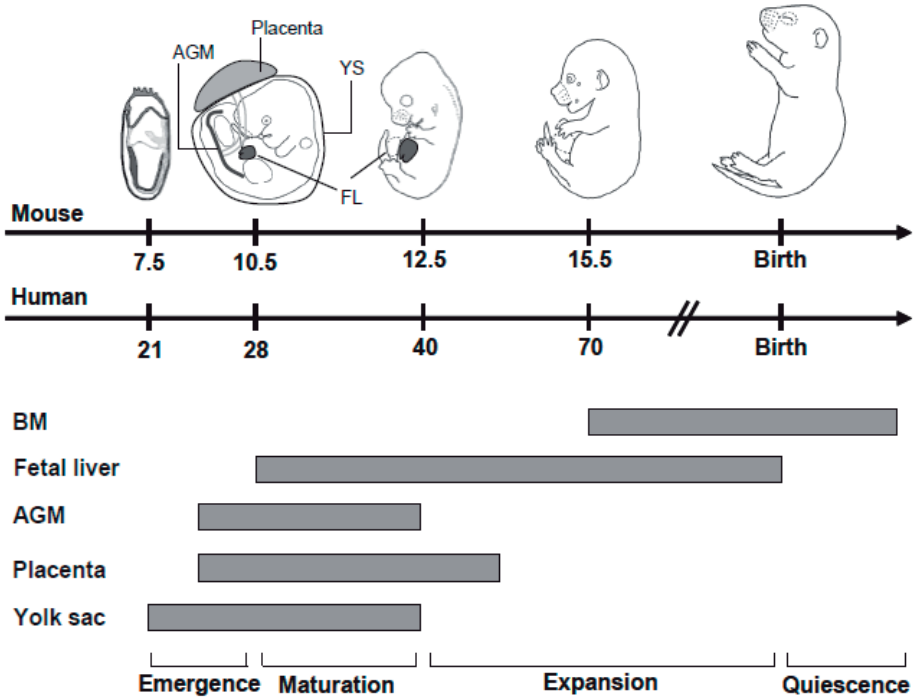

Figure 2. Main anatomical sites of hematopoiesis during embryonic development.

Bars depict the ages at which mouse and human hematopoietic sites are active. AGM, aorta-gonad-mesonephros region; FL, fetal liver; YS, yolk sac.

T cell development in the thymus

T-cell development occurs in the thymus, while all other blood cell lineages develop in the BM. The thymus is a bilobed organ located behind the sternum, above the heart. It is significantly large at birth, but the volume of true thymic tissue decreases by aging, during a process called thymic involution [59, 60]. Organogenesis of the murine thymus starts at E10.5. Bilateral endodermal proliferations of the third pharyngeal pouch invade the underlying mesenchyme to form the thymic primordium or anlage [61]. In humans, this starts at the end of the fourth week of gestation. At E12.5 (4-7 weeks in man), the primordia separate from the pharynx and migrate to their definitive location, where they fuse to form a single organ [62, 63]. The importance of the thymus as an essential microenvironment for T cell development is proven by children suffering from the DiGeorge syndrome, in which sometimes thymus is completely missing. These children have a severely low number of T cells or even a complete absence of T cells [64]. BM hematopoietic progenitors enter circulation and migrate to the thymus where they commit to the T-cell lineage and further maturate to the functional T lymphocytes. Since thymic progenitors lose their self-renewal potential, a continuous import of progenitors from the BM is required to maintain T-lymphopoiesis [65]. However, upon deprivation of thymus from BM derived progenitors the thymus can maintain autonomous T-cell development for several months [66], a turnover process which is regulated by bone marrow progenitor colonization. It has been shown that the thymocyte turnover is regulated by natural cell competition between young BM derived progenitors and old thymus resident progenitors. When the thymus is relieved from outside competition, intrathymic precursors persist, self-renew, resulting in the development of T-ALL Leukemia [33].

T-cell development proceeds through a series of discrete phenotypic stages that can be characterized by the expression of several important membrane molecules, most notably CD4 and CD8 (Figure 3). In both humans and mice, thymocyte development occurs through successive CD4-CD8- (double negative, DN), CD4+CD8+ (double positive, DP) and CD4+CD8-CD3+ or CD8+CD4-CD3+ (single positive, SP) stages. The DN subset can be further subdivided into four stages (DN1 till DN4) in mice and humans [67-69]. The precise identity of the progenitors that seed the thymus is still controversial due to the fact that heterogeneous subpopulations of BM progenitors contain T-cell lineage potential, and also the extremely low number of cells seeding the thymus. These progenitors enter the thymus via veins in the cortical tissue close to the corticomedullary junction, from which they migrate into the thymic tissue [70] (Figure 3). Although few progenitors migrate to the thymus, they significantly proliferate in response to the environmental signals they encounter, while starting a T-cell transcriptional program. These initial signals are provided via cytokines like SCF and Flt3L, Wnt and Notch signaling pathway.

Several types of progenitors have been suggested to seed the thymus and most of them are known to circulate and express chemokine receptors and adhesion molecules, shown to be involved in the thymus migration and seeding. These consist of Ccr7, Ccr9, and the P-selectin ligand Psgl1, among others. Despite several subpopulation candidates of thymus-settling, one major progenitor source contains lymphoid-primed multipotent progenitors (LMPP) [71, 72]. These progenitors are defined as Lin-Sca1+c-Kit+Flt3+ and, besides T-cell potential, they can develop into macrophages, dendritic cells, NK cells, and B-cells, but not erythrocytes or megakaryocytes lineages [73-75], at least in the mouse. In humans, the earliest cells in the thymus have retained some erythroid potential [69], which also is reflected in the abundant erythroid gene program that is expressed in human ETP-ALL [76].

After entering the thymus T-cell precursors develop through distinct stages. Progression through these steps involves gradual phases of lineage specification, characterized by the acquisition of a T-cell specific transcriptional program. Concomitantly to the lineage specification events, T-cell precursors gradually and irreversibly lose alternative non-T lineages potential till they are fully committed to the T-cell lineage. While the B-cell potential is rapidly lost by the majority of the progenitors entering the thymus, the potential to become dendritic cells (DCs), natural killer (NK) cells and macrophages are preserved until later stages. Initial studies identified cells with a CD3-CD4-CD8-CD25-CD44+ surface phenotype (named DN1) as the most immature T-cell progenitors in the thymus. Further studies demonstrated that this is still a highly heterogeneous population also containing mature NK, NKT-cells, and γδ T-cells. In addition, effective T-lineage progenitor activity was shown to reside in a small subset of DN1 cells expressing c-Kit, which is termed early thymic progenitors (ETP) [24, 77-79]. ETPs are very efficient in the generation of DN2 cells (defined as CD3-CD4-CD8-CD25+CD44+) but they still maintain NK, DC, myeloid and at a lower extent also B-cell potential. Similarly to the LMPPs, a small portion of ETPs also expresses Flt3 and CCR9. These are believed to be the most immature T-cell progenitors in the thymus in the mouse.

The presence of alternative lineage potential in T-cell progenitors suggests the existence of mechanisms to dictate a T-cell fate at the expense of other lineages. The most well-known instructive signal to promote T-cell development is the Notch signals. Activation of the Notch signaling pathway by ligands of the Delta family was shown to be essential to induce T-cell commitment [80-84]. While Notch signaling appears to be involved in the restriction of alternative lineage potentials e.g. by inhibiting B-cell and myeloid cell development, it may alternatively promote survival and expansion of the T-cell progenitor populations [85-87]. Another signaling pathway shown to be essential for these early events in T-cell

development is the Wnt signaling pathway, which is currently seen as a rate-limiting positive regulator of the transition to the DN2 stage [88, 89].

T cell progenitors migrate through different anatomical zones in the thymus which may provide different signals to help the establishment of a T-cell development program (Figure 3) [90, 91]. As ETPs migrating through the cortical region towards the subcapsular zone they become more restricted to the T-cell lineage and start expressing important genes for T-cell receptor (TCR) rearrangements, assembly and signaling, such as recombinase activating gene 1 (Rag1) and Rag2, CD3 chains and Lck, [67]. The progressive upregulation of T-cell identity genes is accompanied by the acquisition of a DN2 (CD3-CD4-CD8-CD25+CD44+) and DN3 (CD3-CD4-CD8 CD25+CD44-) surface phenotypes, a process called T cell commitment. T cell commitment process has been studied more extensively which resulted in the identification of more intermediate stages namely DN2a, DN2b, DN3a, and DN3b. DN2a T cells also express a high level of c-kit and are believed to be the last uncommitted stage of T cell development. The expression of c-kit diminishes significantly at the DN2b stage while they lose their capacity to differentiate into any non-T cell lineage anymore [92]. CD27 expression subdivides DN3a and DN3b pre- and post-selection DN3 cells respectively. Detailed gene expression analysis revealed that regulatory changes associated with the β-selection occur between these two stages of DN3 [93]. The DN3a stage is characterized by an arrest in cell-cycle allowing in this way the rearrangement of the Tcrb genes, which encodes for the variable region of the antigen receptors in T-cells. These rearrangements occur through a process termed V(D)J recombination which allows the generation of a high diversity of antigen receptors [94]. Successful rearrangement of the Tcrb gene is functionally tested for its expression on the cell membrane. Productively rearranged Tcrβ chains are coupled to an invariant pre-Tα chain to form the Pre-TCR complex. Signaling through the Pre-TCR induces proliferation, survival and differentiation, in a process called β-selection. Cells that pass the β-selection are educated to develop into the αβ-T cell lineage [95, 96] and acquire DN4 (Thy+CD3-CD4-CD8-CD25-CD44-), ISP (CD3-CD4-CD8+ in mice or CD3-CD4+CD8- in humans) and later DP (CD4+CD8+) surface phenotypes. After these highly proliferative stages, another arrest in proliferation happens when the cells reach the DP stage and start rearranging the Tcra gene. Efficient Tcra rearrangement leads to the expression of a TCRαβ complex on the cell surface. These TCRαβ complexes are then functionally tested for the recognition of self MHC (major histocompatibility complex) molecules (positive selection) and absence of reactivity against self-antigens (negative selection) [97]. Therefore this stage is identified by high apoptosis rate in order to eliminate non-functional and auto-reactive T-cells [97, 98]. Concurrently with the positive and negative selection processes, cells with a functional T-cell receptor further maturate to CD4+ T-helper cell or to CD8+ cytotoxic T cell lineages and migrate to the periphery.

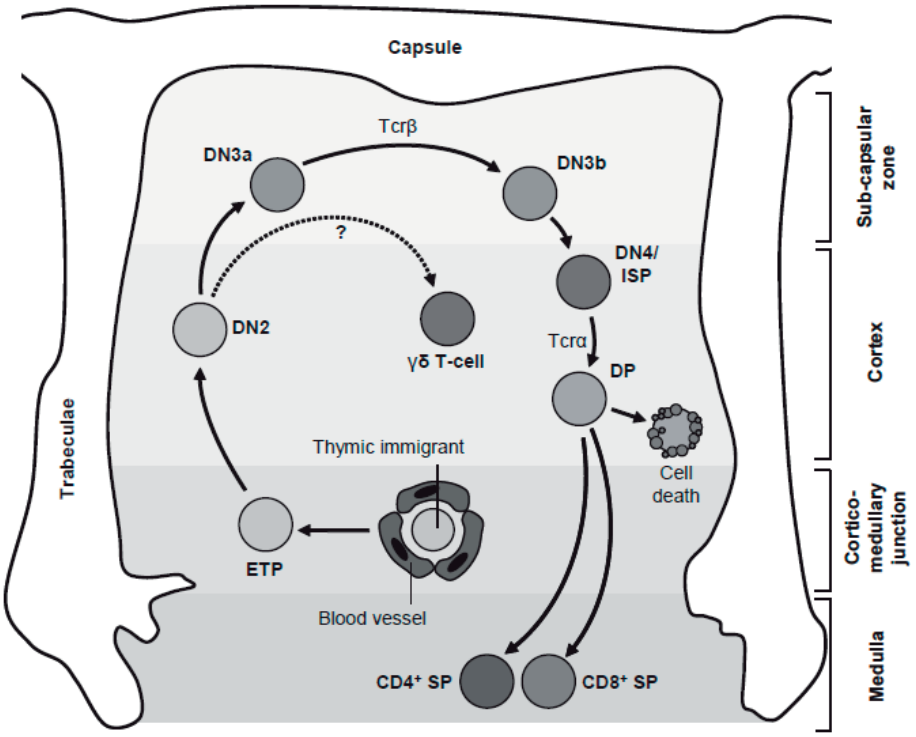

Figure 3. T cell developmental stages in the thymus.

Cross-section of an adult thymic lobule representing the migration route of T-cell precursors during development. Immigrant precursors move to the thymus through blood vessels and enter near the cortico–medullary junction, the early T-cell precursors (ETP) subsequently migrate, and differentiate to double negative (DN), double positive (DP) and finally to single positive (SP) stages, through the discrete microenvironments of the thymus. β-selection occurs at DN3a to DN3b transition at the outer portion of the thymus (subcapsular zone). A directional reversal of migration back across the cortex towards the medulla occurs for the later stages of thymocyte development. ISP, immature single positive; TCR, T-cell receptor.

Wnt signaling pathway

The terminology of Wnt originates from a combination of the names for the Drosophila melanogaster segment-polarity gene Wingless, and Integrase-1 [99], a mouse proto-oncogene that was discovered as an integration site for mouse mammary tumor virus. Integrase-1 is the vertebrate homolog of D. melanogaster Wingless, suggesting a key role of the gene in carcinogenesis [100]. There are 19 Wnt genes in the human and mouse genomes, all encoding lipid-modified secreted glycoproteins.

The involvement of Wnt signaling pathway in various developmental processes has been shown by many studies. Among all cell-fate specification, progenitor-cell proliferation, dorsal axis development, and control of asymmetric cell division are the most important processes. In the hematopoietic system, Wnt pathways also play an important role as a proliferative growth factor, but also to determine cell-fate decisions, as morphogens do in other tissues. The number of studies on the Wnt signaling by immunologists and hematologists has augmented dramatically during the past few years. Initially, the pathways were only fascinating for developmental immunologists where it was thought that Wnt signaling is only important during T cell development by providing proliferative signals to immature thymocyte. Nowadays, Wnt signaling is also a hot topic in the field of immune-hematology where the role of Wnt signaling is heavily under investigation e.g in the self-renewal of hematopoietic stem cells, the maturation of DCs, peripheral T-cell activation and migration, and the development of leukemias.

There are at least three different Wnt pathways: the canonical Wnt pathway, which involves β-catenin (also known as cadherin-associated protein-β) and members of the T-cell factor (Tcf)/ lymphocyte-enhancer binding factor (Lef) family; the planar cell polarity (PCP) pathway; and the Wnt-Ca2+ pathway.

Canonical Wnt signaling

Canonical Wnt pathway, known as β-catenin dependent Wnt signaling, is the most studied and best defined Wnt pathway. The majority of studies in immune-hematology have focused on this pathway in which β-catenin is the central player [101, 102] (Figure 4). This 92 kD protein has two functions: cell adhesion via cadherin, and activator of the canonical Wnt pathway.

In the absence sufficient triggers in the environment, the pathway is off. As a result, free cytoplasmic β-catenin is kept at very low levels via proteasomal degradation. β-catenin degradation is achieved through active phosphorylation at conserved regions by the ser/thr kinases glycogen synthase kinase 3β (Gsk-3β) and Casein Kinase 1 (Ck1). These proteins consist the so called destruction complex, that also includes the scaffolding proteins axis inhibition protein 1 and 2 (Axin1 and Axin2), and the tumor suppressor protein adenomatous polyposis coli (Apc). First, β catenin is phosphorylated on Ser45 by Ck1, and then on Ser33, Ser37 and Thr41 by Gsk-3β to form recognition sites for the ubiquitin ligase β-transducin repeat-containing protein (β-Trcp), resulting in its ubiquitylation and subsequent proteasomal breakdown [103] (Figure 4A).

In the presence of surrounding Wnt proteins, and upon binding of these soluble proteins to the Frizzled receptor and the coreceptor low-density lipoprotein receptor-related

protein 5 (Lrp5) or Lrp6 at the cell membrane, the signaling cascade is induced. When the complex of Frizzled–Lrp5/Lrp6 is formed, the ser/thr kinases is inhibited which is mediated by Dishevelled (Dlv). This phenomenon results in the disruption of the destruction complex, thereby β-catenin is stabilized in the cytoplasm. Accumulation of β-catenin probably in its amino-terminally dephosphorylated form [104] is followed by translocation to the nucleus where it binds to Tcf/Lef transcription factors. In normal conditions, Tcf assembles a transcriptional repressor complex [105]. Formation of the active β-catenin/Tcf transcription-factor complex induces upregulation of Wnt target genes particularly Axin-2, c-Myc, and cyclinD1 (Figure 4B). Recent biochemical studies have revealed dual roles for Gsk-3β and Ck1. It has shown that they function not only as a negative regulator by promoting β-catenin phosphorylation and degradation, but also function as a positive regulator. By phosphorylation of specific residues of Lrp6, they allow docking of Axin to be rescued from the destruction complex. Therefore, despite their inhibitory form in cytosolic forms, membrane associated Gsk-3β and Ck1 stimulate Wnt signaling upon activation of the pathway [106-108].

The canonical Wnt pathway is known to be strictly regulated at different levels (reviewed in [109]). Binding of Wnts to the receptor complex can be actively prohibited by naturally occurring soluble decoy receptors such as secreted Frizzled-related protein (sFrp) and Wnt inhibitory factor 1 (Wif1) [110]. This Wnt antagonist binds directly to soluble Wnt proteins in order to inhibit their binding to the Wnt receptor complex. Next group of soluble Wnt antagonist consists of the Dickkopf homologs (Dkk), which bind to the Lrp5/Lrp6 co-receptors and inhibit their function. In addition, Dkks can interact with another type of transmembrane receptors, the Kremens (Krm). Krm/Dkk1/Lrp6 form a ternary complex that disrupts Wnt/Lrp6 signaling by promoting endocytosis and removal of the Wnt receptor from the membrane [111]. The first evidence that Wnt signaling is important in stem cells originated from a Tcf-4 knockout experiment in which mutant mice do not develop crypt stem cell compartments. Gene expression analysis revealed that Lgr5/Gpr49 (target of Tcf4 gene) is highly expressed in crypt stem cells, same as multiple other tissues [112]. Parallel studies showed that R-spondin receptor family, which acts as a Wnt agonist, provide growth stimuli for these crypts. The story got completed with the finding that Lgr5 constitutes the receptor for R-spondins in a complex with Frizzled/Lrp [113]. The Lgr5/R-spondin complex acts by neutralizing Rnf43 and Znrf3, two transmembrane E3 ligases that remove Wnt receptors from the stem cell surface. Rnf43/Znrf3 are themselves encoded by Wnt target genes and constitute a negative Wnt feedback loop [114]. In the nucleus, the cell autonomous inhibitor of β-catenin and Tcf (ICAT) prevent the interaction of β-catenin with Tcf and Lef molecules, thereby inhibiting assembly of the active bipartite transcription-factor complex [115]. At least eight isoforms of Tcf with different potential for binding to β-catenin are created by alternative splicing and promoter usage, thereby

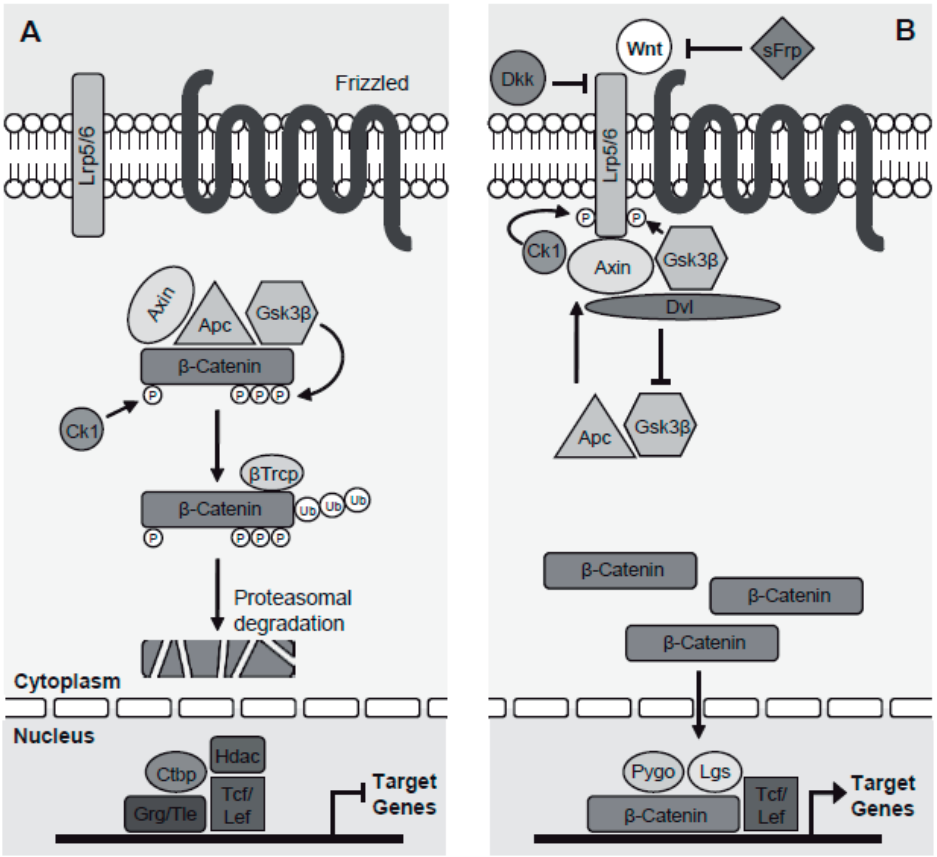

Figure 4. Canonical or Wnt-β-catenin-Tcf/Lef signaling.

A) When the Wnt signalling is off, β-catenin levels in the cytoplasm and nucleus are kept low due to the continuous phosphorylation by the serine/threonine kinases Ck1 (casein kinase 1) and Gsk3β (glycogen synthase kinase 3β), leading to binding of β-transducin-repeat-containing protein (βTrcp) and to ubiquitylation and degradation by the proteasome. The destruction complex is composed of Ck1 and Gsk3β, as well as the anchor proteins Axin1 (axis inhibition protein 1) and Apc (adenomatous polyposis coli). In the nucleus, Tcf (T-cell factors) are bound by co-repressors such as Grg/Tle (Groucho/transducin-like enhancer) proteins that silenced expression of Wnt target genes. Other components of the repressor complex include CTbP (C-terminal binding protein) and Hdac (histone deacetylases). β-catenin in the nucleus is prohibited from binding to Tcf by ICAT (cell autonomous inhibitor of β-catenin and Tcf). The Frizzled receptor complex (composed of Frizzled and Lrp5 (Ldl receptor related protein 5) or Lrp6) can also be actively inhibited by receptor-bound soluble inhibitors such as Dkk1 (Dickkopf homolog 1). B) Upon binding of a lipid-modified Wnt protein to the receptor complex, a signaling cascade is triggered. LRP is phosphorylated by Ck1 and Gsk3β, and Axin1 is recruited to the plasma membrane. The kinases in the β-catenin destruction complex are inactivated and β-catenin translocate to the nucleus to form an active transcription factor complex with Tcf, results in transcription of a large set of target genes. In the nucleus, β-catenin binds to Tcf and Lef factors and recruits co-factors such as legless (Lgs; also known as Bcl9) and Pygopus (Pygo), Cbp/p300, Brahma and Med12/mediator to initiate transcription. Dvl, mammalian homologue of Drosophila Dishevelled.

manipulating the responsiveness of cells to canonical Wnt signals [116]. While the longer Tcf isoforms contain the amino-terminal catenin-binding domain, the shorter forms lack this region, therefore, cannot bind to catenin and function as naturally occurring repressors of the pathway.

The target genes which could be activated by the canonical Wnt signaling pathway are not completely discovered. These genes could vary among different tissues. In general, Axin2, c-Myc, CyclinD1, n-Myc, Lef1, and Cd44 have revealed to be regulated by Wnt signaling in various tissues [117-120]. These genes are involved in cell cycle regulation, apoptosis and proliferation, and in the induction of the Wnt signals in positive feedback loop.

Non-canonical Wnt signaling

As mentioned previously there are other types of Wnt signaling pathways which are independent of β-catenin so called non-canonical Wnt pathway, among all the Planar Cell Polarity (PCP) and the Wnt-Ca2+ pathways are best defined in model organisms such as D. melanogaster, Xenopus laevis and C. elegans, and have been shown to affect hematopoiesis and lymphopoiesis [121, 122].

Binding of non-canonical Wnt ligands such as Wnt5a and Wnt11 to Frizzled and Dvl (and probably G-proteins), without the involvement of Lrp5 and Lrp6 coreceptors, induce PCP signaling [123] (Figure 5). It has been shown that downstream pathways of Dvl are involved in various mechanisms. For example Dvl indices cytoskeletal re-organization by activation of Daam (Dishevelled associated activator of morphogenesis), subsequent induction of the RhoA (Ras homolog gene-family member A)-Rock (Rho-associated coiled-coil-containing protein kinase) pathways [124]. Dvl also activates Rac1, and both of these small GTPases (RhoA and Rac1) activate the Jun N-terminal kinase (Jnk, stress-response) pathway, which affects the cytoskeleton and cell shaping. Other important roles of the PCP pathway are the positional adjustment in model organisms, cell adhesion, and migration by regulating the cytoskeleton modification, and blockage of canonical Wnt signaling in lymphocytes by phosphorylation of cytoplasmic β-catenin protein [121, 122]. Several downstream elements of the PCP pathways affect the actin cytoskeleton and cell polarity, although this is not supposed to be true during hematopoiesis.

Binding of non-canonical Wnt5a ligand to e.g. Frizzled-2 receptor could trigger yet another pathway, known as Wnt-Ca2+ pathway which could inhibit canonical Wnt pathway [125, 126]. The Wnt-Frizzled binding via a G protein, activates phospholipase C (Plc), leading to the cleavage of phosphatidylinositol-4,5- bisphosphate (PtdIns(4,5)P2) to inositol trisphosphate (InsP3) and diacylglycerol (DAG) (Figure 5), which activates protein kinase C (PKC). InsP3 binding to its receptor on intracellular calcium storage cause accumulation

of the cytoplasmic level of Ca2+ ions, which is low in normal conditions. The elevation of Ca2+ concentration induces the phosphatase calcineurin and several calcium dependent kinases, including PKC (Protein Kinase C). Pkc itself upregulate the calcineurin expression, which ultimately leads to the Nfat activation (nuclear factor of activated T cells). In Xenopus spp., the Wnt-Ca2+ pathway has been shown to control ventral patterning, partially via Xenopus Nfat. Nfat is known to be involved in T-cell receptor (TCR)-mediated activation and interleukin-2 (IL-2) production in T cells which could be regulated via Wnt-Ca2+ downstream components. [127, 128]. Another interesting association of Wnt and Nfat signaling has been described in T cells where Gsk3β play a role in exporting Nfat out of the nucleus [129].

The presence of nineteen mammalian Wnt ligands which can bind to 10 different FZDs receptors to activate various downstream pathways such as WNT/β-catenin, WNT/planar cell polarity, and WNT/Ca2+ pathway, make the study of this pathway complicated. In the last decade, various in vitro and in vivo gain of function and loss of function models have been developed to unravel these functions. Nevertheless, our knowledge at the cell membrane level where Wnt ligands activate FZD receptors, to trigger specific mechanisms and associated components, and as result to determine the ultimate function of Wnt pathway is yet incomplete. First of all, the level of WNT/ FZD interaction is generally unknown in particular in mammals, and it is unclear which WNT/FZD combinations can activate certain signaling pathways. Secondly, selective binding of a specific FZD receptor to downstream signaling pathways is not fully understood. However, the binding of several WNTs/FZDs complex and the physiological outcome of this interaction has been the subject of several studies which are reviewed in [130], and some of the most important ones will be mentioned here. FZD2 triggers both canonical and non-canonical Wnt-Ca2+ pathways. WNT-5A has been shown to bind to FZD3, triggering non-canonical WNT pathway. WNT 5A also triggers β -catenin signaling in HEK293 cells via FZD4, which can be inhibited when it is cotransfected with the non-canonical receptor ROR2. WNT-7A and WNT-3A bind to FZD5/CRD, as shown by an ELISA-based protein binding assay. Some studies have identified FZD6 as a negative regulator of the β-catenin dependent pathway. The binding of WNT-3 and FZD7 results in the increased stabilization of β -catenin in human hepatocellular carcinoma cells and might play a role in cancer development. Purified and secreted forms of FZD5, FZD7, and FZD8 CRD region has shown to antagonize WNT-3A-induced β -catenin accumulation in L-cells, whereas, in mouse embryonic stem cells, the same CRDs can inhibit spontaneous mesoderm development and induce neural differentiation. Lastly, it has been shown that FZD10 is involved in the development of colorectal cancer, mainly through canonical Wnt pathway.

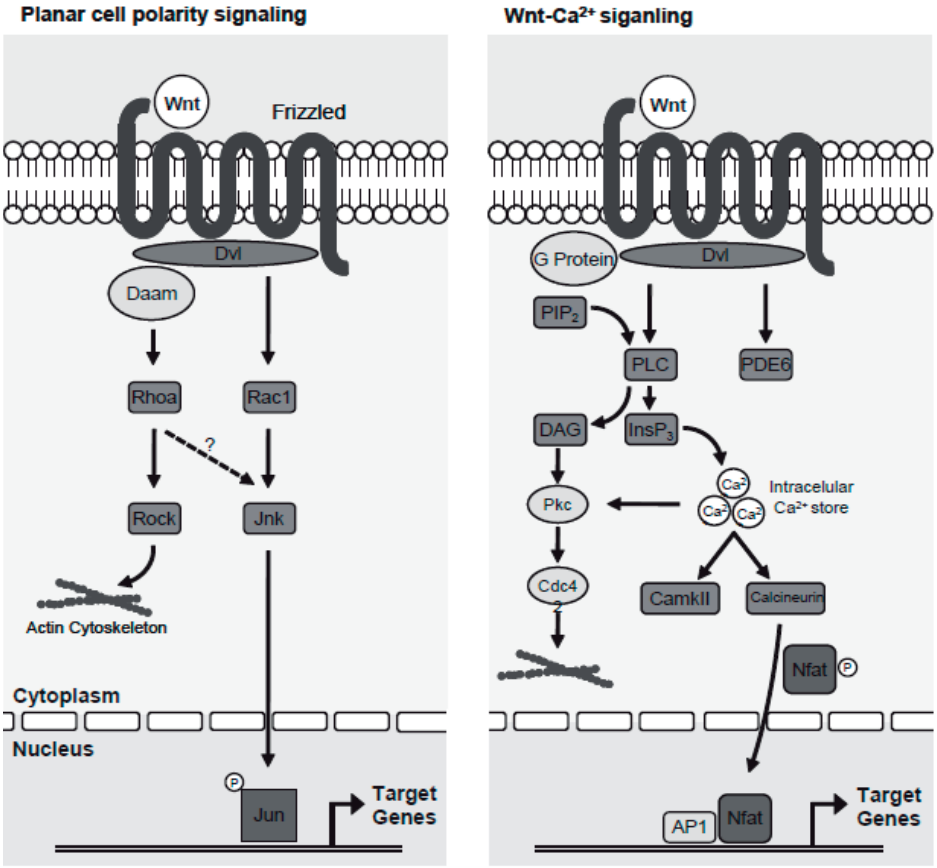

Figure 5. Non-canonical Wnt signaling.

A) Planar cell polarity (PCP) signaling does not involve β-catenin, Lrp (LDL receptor related protein) or Tcf (T-cell factors), but leads to the activation of the small GTPases RhoA (Ras homologue gene-family member A) and Rac1, which upregulate the stress kinase Jnk (Jun N-terminal kinase) and Rock (Rho-associated coiled-coil-containing protein kinase 1) and result in remodelling of the cytoskeleton and modifications in cell adhesion and motility. Through largely unknown mechanisms, canonical β-catenin signaling can be inhibited by the PCP pathway. B) Wnt-Ca2+ signaling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the kinases PKC (protein kinase C) and CamkII (calcium calmodulin mediated kinase II) and the phosphatase calcineurin. The activation of Plc (phospholipase C) by Dvl (mammalian homolog of Drosophila Dishevelled) leads to the cleavage of PtdIns(4,5)P2 (phosphatidylinositol bisphosphate) into InsP3 (inositol trisphosphate) and DAG (diacylglycerol). DAG, together with calcium, activates PKC, whereas InsP3 binding to receptors on the membranes of intracellular calcium storage causes a transient increase in cytoplasmic free calcium, often also inducing an increase from extracellular supplies. AP1, activator protein 1; Cdc42, cell-division cycle 42; Daam, the Dishevelled-associated activator of morphogenesis; Nfat, nuclear factor of activated T cells; Pde6, phosphodiesterase 6.

Tyrosine kinase receptor and their relation with Wnt signaling

Some of the Receptor Tyrosine Kinase (RTK) family members including, Ryk (related to receptor tyrosine kinase), Ror (RTK-like orphan receptor), and MuSK (muscle-specific kinase) have an extraordinary connection to Wnt signaling pathway, thereby accounts for Wnt signaling receptors [131]. Ryk protein consists of a Wnt-inhibitory factor-1 (WIF-1) domain in its extracellular region and is reported to function as a receptor (or co-receptor) for Wnts [132] (Figure 6). The role of Ryk was initially discovered during screening for genes involved in Drosophila CNS axon pathfinding [133]. For a long time, it was believed that Ryk does not contain active tyrosine kinase catalytic activity because of the mutations in the vital ATPase sites. A breakthrough in understanding Ryk function stem from the study in Drosophila where binding of the WNT5 protein to Ryk receptor has been shown [134]. It turned out that the Ryk-WNT5 interaction triggers the development of the embryonic CNS [135]. Dr. Baltimore and his coworkers have shown that Ryk can also directly binds to Wnt-1 and Wnt-3a via its WIF domain. This binding induces a canonical downstream pathway which results in TCF activation. In this in vitro model, the extracellular domain of Ryk forms a ternary complex with Frizzled and Wnt-1. The intracellular domain of Ryk interacts with Dishevelled, in order to induce TCF activation in response to Wnt-3a activation. They have shown that Ryk-Wnt3a interaction is crucial for the development of neurite outgrowth in dorsal root ganglia explants [136].

The majority of functional studies focus on the role of Ryk in CNS development during embryogenesis. There are only a handful of studies in which the roles of Ryk in hematopoiesis and thymopoiesis have been studied. More than 20 years ago, the laboratory of Dr. Belmont studied the expression pattern and function of Ryk in the hematopoietic system. Gene expression, as well as protein expression analysis, revealed that Ryk expression is regulated during hematopoietic development by lineage commitment and stage of maturation [137]. After two decades, a study in the laboratory of Prof. Nemeth revealed that Wnt5a ligand induces HSC quiescence through a non-canonical Wnt signaling pathway, which leads to an increased reconstitution after transplantation. Their further investigation demonstrated that Wnt5a regulates HSC quiescence and hematopoietic repopulation via binding of Wnt5a to the Ryk receptor, a process in which suppression of reactive oxygen species (ROS) is required [138].

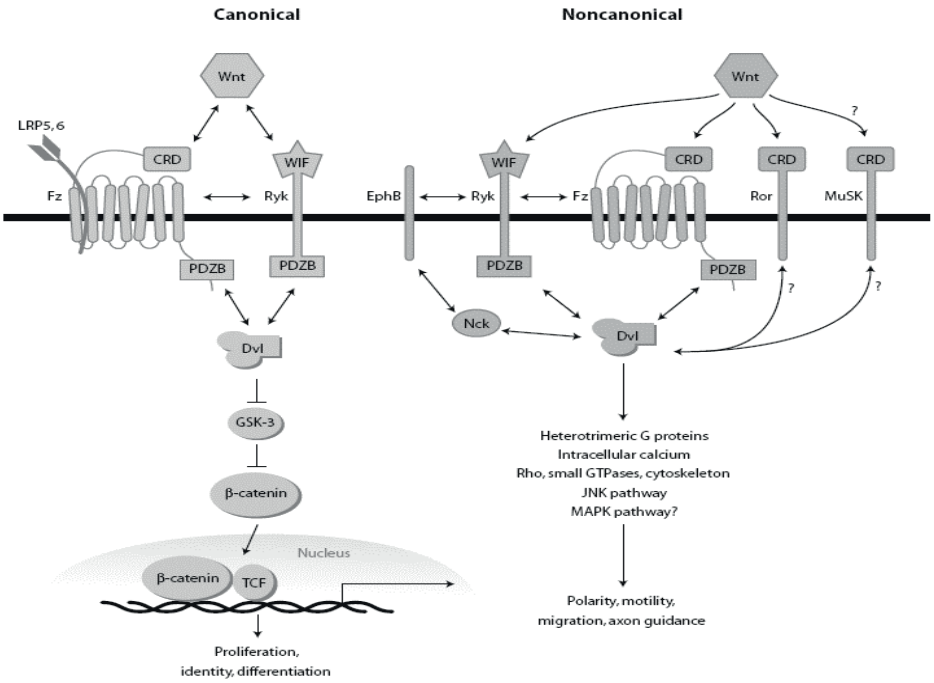

Figure 6. Cooperative binding of Ryk to Wnt, Dvl, or adaptor and scaffold proteins.

Many different transmembrane proteins that participate in Wnt signal transduction have now been identified. Domains important for Wnt binding (CRD in Fz, Ror, MuSK; WIF in Ryk) or for Dvl binding (PDZ-binding motif "PDZB") are shown. Fz, LRP5, or LRP6 are essential for canonical Wnt signaling; there is evidence that Ryk contributes to this in some contexts. Ryk and Fz have also been implicated in various non-canonical signaling processes. Similarly, several other receptor proteins may participate on the basis of proposed Wnt-binding domains, established protein-protein interactions, or both. Double-headed arrows indicate biochemically confirmed direct or indirect associations. Conjectural associations are denoted with a question mark. Other crucial interactions between these proteins not discussed in the text (e.g., between the Dvl DEP domain and Fz in non-canonical signaling) are not shown.

Roles of Wnt signaling in hematopoiesis

Several reports have shown the importance of canonical and non-canonical Wnt signaling in HSC biology. Today, a large body of evidence has proven that Wnt signaling plays crucial roles in the self-renewal of HSCs and proliferation of progenitor cells. However, several questions are not fully addressed yet. For example, under which physiological condition, e.g. homeostasis or proliferation, Wnt signaling is crucial, or what is the optimal dosage of Wnt signaling activity for HSC repopulation, and which transcriptional activator is crucial in this process.

First evidence for the involvement of Wnt signaling during hematopoiesis stem from the gene expression data where differential expression level of several Wnt signaling

components in LT-HSC, primitive progenitors, and BM niche compartments, both in mice [139] and humans [140], and in adults as well as in fetal hematopoietic organs have been observed. These expression patterns include both paracrine and autocrine effects of Wnt ligands [141]. Wnt5a, Wnt2b, and Wnt10b have shown to trigger in vitro proliferation of human HSCs and primitive progenitors, in order to maintain an immature phenotype and sustain HSC pool. On the other hand, in vitro reporter assays have shown that murine HSCs residing in the BM niche are responsive to the canonical Wnt signals due to high expression Wnt receptors, and induce -β-catenin-Tcf/Lef downstream pathway [142]. Induction of canonical Wnt signaling by using purified Wnt3a results in an increased reporter activity in Bcl2 (B-cell lymphoma 2)-transgenic LSK cells and thereby enhanced self-renewal of the cells [143]. Consistently, blockage of this pathway through ectopic expression of Axin1 or by using a truncated form of Frizzled leads to a reduction of HSC proliferation in vitro and subsequently diminished repopulation potential of the transplanted cells in vivo [142]. Another evidence on the role of canonical Wnt signaling in maintenance of HSC pool comes from a gain of function study in which overexpression of constitutively active β-catenin in lymphoid or myeloid progenitors produced uncommitted cells with multilineage differentiation potential [144].

An important investigation carried on in our laboratory in which a mouse model with a germline mutation specifically in the Wnt3a gene was used. This study has shown that Wnt3a canonical signaling is essential for self-renewal of fetal liver HSCs. Of interest, Wnt3a deficiency effect on HSCs could not be substituted by any other Wnt protein expressed in fetal liver and resulted in the full inhibition of the canonical Wnt signaling pathway, indicating that Wnt3a plays a non-redundant role in the regulation of fetal liver HSC function. The fact that other expressed Wnt proteins in fetal liver, are not able to compensate for Wnt3a deficiency suggests that either only Wnt3a is present specifically in the niche or exact ligand-receptor complex containing Wnt3a is required for fetal liver HSC expansion [145]. Another study from our laboratory unraveled the dosage dependency of HSCs on canonical Wnt signaling pathway. In this study Luis et al. used different transgenic mouse lines carrying different combinations of targeted mutations of the negative Wnt signaling regulator Apc, thereby a gradient of different levels of Wnt signaling activation was generated. Controlled limiting-dilution competitive transplantation assays demonstrated that only a mild levels of Wnt signaling upregulation, approximately 2 fold higher than the normal physiological levels, leads to an enhanced HSC activity and increased repopulation. However, intermediate to high levels of Wnt signaling activity cause lack of HSC repopulation in recipient mice [146].

Crucial signaling pathways in thymopoiesis

Notch signaling

Hematopoiesis and thymopoiesis, like other developmental processes, require a strict spatial and temporal control and harmonized gene expression programs. The majority of lineage commitment events in metazoans are controlled by merely a few signaling pathways including Wnt, Notch, TGF-β, Hedgehog, and receptor tyrosine kinases (RTK). Each pathway is frequently used in several processes, activating diverse subsets of target genes in various developmental contexts.

The Notch signal transduction pathway is not unique to developing T cells, but in the development of blood cells, its most prominent role is to induce a T cell gene program in mulita potent progenitor cells that arrive in the thymus [1]. In many other tissues and organs, Notch signaling similarly regulates cell fate determination. Notch signaling involves cell-cell interactions, rather than binding of a soluble ligand to a receptor. There are four Notch receptors, named Notch-1 to 4. Signaling is initiated when the large extracellular domain of the Notch receptor binds a membrane bound ligand on a neighboring cell. The five Notch ligands in mammals are Delta-like-1, 3 and 4 and Jagged-1 and 2. Delta-like-2 is a non-expressed pseudogene.

Interaction of Notch with a ligand induces proteolytic cleavage of the intracellular part of Notch (intracellular (IC-) Notch). IC-Notch then translocates to the nucleus and binds to the nuclear transcription factor CSL (CBF1 stands for core-promoter binding factor in humans, suppressor of hairless in Drosophila, Lag-1 in Caenorhabditis elegans; also called RBP-k in mouse). Binding of IC Notch to CSL induces the dislocation of co-repressors (coR) such as Mint and Nrarp, and recruitment of coactivators (coA), such as Mastermind (Maml), consequently leading to activation of Notch target genes [147]. In the thymus, there is an abundant expression of DLL-4, the Notch ligand that is mostly responsible for inducing a T cell lineage program on developing thymocytes [148]. Indeed Notch-1 KO mice show an arrest in T cell development at the DN1 stage with a relative increase of B cells in the thymus [84]. Conversely, ectopic expression of IC-notch in the BM leads to T cell development in the BM niche with DP cells containing Tcrb rearrangements [83].

Wnt signaling

Historically, the importance of Wnt signaling in the hematopoietic system was first documented from the effect of Wnt signaling during T-cell development in the thymus (Figure 7). This was due to the observation that thymic epithelial cells (TEC) express a high level of Wnt proteins including Wnt3a and Wnt5a. Soluble Frizzled receptors which were used as decoys for Wnt proteins showed inhibition of thymocyte differentiation in fetal thymic

organ cultures (FTOC), an in vitro assay for T-cell development, mainly by blocking thymocyte proliferation [149]. Consistent with this study, the thyme of Wnt-1 and Wnt-4 double KO mice have a low thymocyte cellularity [150]. These observations were followed and more highlighted by other sequential studies showing the lack of T- and B-cell development in mice deficient for the Wnt-responsive transcription factors Tcf-1 and Lef-1, respectively [151, 152]. At early ages, Tcf-1 KO mice have an incomplete block at the DN1, DN2 and ISP stages of thymocyte development, whereas mature mice have a full block at the DN1 stage (Figure 7). Although Lef-1 deficient mice have a normal thymopoiesis, Tcf-1 and Lef-1 double KO mice have a complete block at the ISP stage, indicating redundancy in function of these transcription factors during thymopoiesis. A key observation was that Tcf-1 and Lef-1 are capable of binding to β-catenin and subsequently turn them into activator transcription factors [116, 149, 150, 153-155], which is consistent with the finding that Wnt signaling provides proliferative signals to immature T and B cells [149, 150, 155].

Canonical Wnt pathway

As the number of progenitors seeding the thymus is limited, an enormous expansion of cells takes place during the early phase of T cell development. Cytokines, mainly IL7, but also Wnt proteins, are responsible for the initial proliferation of thymocytes before β-selection. Indeed, it was previously shown that Wnt proteins are secreted by thymic stromal cells, and canonical Wnt signaling is most active in the most immature DN stages [156]. In this regard, it is fitting that the first T cell specific target gene of the Notch pathway to be induced is Tcf-1 (encoded by the gene confusingly named Tcf7), the nuclear protein responsible for transmitting the nuclear response to Wnt signals. In this way, the Notch pathway starts a positive feedback loop in which T cell specific signal is amplified by Wnt signaling via Tcf-1. Our group has shown that especially early T cells are “hard wired” to respond to Wnt signals. As this differential responsiveness to Wnt signaling during different thymocyte stages is not caused by altered expression of Frizzled or Wnt proteins, but by increased expression of positively acting canonical Wnt factors (such as β-catenin) and decreased expression of inhibitory molecules (such as Axin1) in early DN thymocytes. [156]. Consistently, ICAT overexpression, a negative regulator of Wnt signaling, which blocks the interaction of β-catenin and Tcf/Lef transcription factors, partially inhibit early stages of T cell development but does not alter the later stages. The secreted Wnt inhibitor Dkk1, which blocks binding of Wnt proteins to the required Lrp co-receptor, inhibits thymocyte development similarly at the DN stages. This inhibition is dose dependent such that high levels of Dkk1 cause complete block at the very early DN1 stage of T cell development (Figure 7).

Wnt transduction in thymocytes by overexpressing activated forms of β-catenin caused enhanced thymocytes development [157], as a result of bypassing the requirement for pre-TCR signals in mice lacking a pre-TCR [158, 159], and upregulation of proliferation-as-

sociated genes in immature thymocytes [107]. Notably, conditional T-cell-specific deletion of β-catenin, using the proximal Lck promoter to control Cre expression, impaired T-cell development at the β-selection checkpoint, leading to a predominant decrease of mature T cells in circulation [160]. Another elegant study on the effect of Wnt signaling through β-catenin and Tcf is provided by using transgenic or retroviral reconstitution of Tcf1 KO mice with various isoforms of Tcf1 that can or cannot bind to β-catenin [161]. Only the forms of Tcf1 with the capability of binding to β-catenin could rescue T-cell development in Tcf1-deficient mice, which is consistent with an important role for canonical Wnt signaling through β-catenin in the early stages of T-cell development in the thymus. On the other hand, conditional deletion of the Apc tumor-suppressor gene, inhibits T-cell development, partially by affecting β-catenin signaling [162]. Conditional deletion of β-catenin only in thymocytes disrupts T cell development, suggesting that thymocyte development requires higher levels of Wnt signaling. Consistently, measurement of Wnt signaling activity with in vivo reporter assays [146] showed a significant difference between bone marrow stem/progenitor cells and thymocytes (approximately 4 fold higher in thymocytes) in terms of Wnt signaling activity.

As an approach for understanding the controversies in the literature on Wnt signaling in HSCs, our group reported combinations of targeted mutations in Apc that were used in order to obtain a gradient of Wnt signaling activation. As mentioned previously, while HSC function was enhanced specifically with mild levels of Wnt signaling activity, only intermediate Wnt activation confers an advantage to the early stages of T-cell development. High and very high levels of Wnt signaling activation similarly to stabilization of β-Catenin, resulted in the accumulation of DN3 thymocytes and in impaired Tcrb gene rearrangements [146]. Despite the severe reduction in Tcrb gene rearrangements, Tcrβ- DP and SP cells could be detected in the thymus of these mice, although in reduced numbers, indicating that high Wnt signaling allows a bypass of the β-selection checkpoint. The reduced numbers of DP and SP thymocytes are probably due to the lack of proliferation and survival stimuli from a functional pre-TCR [158, 162]. In contrast, an intermediate activation of the Wnt pathway enhanced early stages of T-cell development while preserving Tcrb rearrangements and maintaining developmental checkpoints. However, the numbers of DP cells was still reduced which may indicate that later stages of T-cell development have different Wnt signaling requirements [163]. In the agreement, in vitro cocultures with OP9 stromal cells expressing the Notch ligand Delta-1, in which positive and negative selection processes are less stringent [164] an increase in both DP and SP cells with intermediate but not higher levels of Wnt activation was observed. In summary, HSCs and thymocytes require different levels of Wnt activation with the thymocytes displaying higher Wnt activity.

Lack of Wnt3a also causes deficiency during T-cell development in murine due to a block at ISP to DP transition which results in decreased total cellularity [145]. The observation is because of deficiency in Wnt3a production by the thymic stroma since Wnt3a KO progenitors stem cells differentiate normally in wild-type thymic lobes. This is in agreement with the fact that Wnt3a gene expression being restricted to the murine thymic epithelium [156]. The similarity between Wnt3a and Tcf1 deficiency phenotype in terms of thymopoiesis suggest that Wnt3a is directly regulating thymocytes development. These phenotypic similarities were later observed in Wnt1 and Wnt4 KO mice suggesting high functional redundancy among these Wnt proteins in the thymus.

It has been shown that canonical Wnt signaling is not only playing roles at early stages of T cell development but also is playing significant roles during positive and negative selection and the DP to SP transition. As some examples, it has shown that DP to CD4+ SP transition is regulated partially by β-catenin-Tcf signaling [165] and that Tcf1 KO mice have diminished expression level of CD4 on DP and CD4+ SP cells (Figure 7). A series of elegant experiments indicate that overexpression of stabilized β-catenin controls the positive selection of thymocytes [166, 167]. Full and simultaneous positive and negative selection of both CD4+ and CD8+ SP thymocytes only happened when stabilized β-catenin was overexpressed, in contrast to the normal thymocyte development where the differentiation of CD8+ SP thymocytes lags behind of CD4+ SP thymocytes (Figure 7).

The thymic microenvironment provides signals that are crucial for thymopoiesis. Although the lymph-node microenvironment produces similar signals, lymph node-derived progenitors are not able to develop into mature T-cells when they are cultured with stromal cells that express the Notch ligand Delta-1. Lymph node stromal cells can generate most of the important signals for T-cell development, including IL-7, SCF, and the Delta1. However, they do not express Wnt transcripts [168]. Interestingly, Lymph-node T-cell progenitors can develop to mature T cells when they are cultured with stromal cells expressing Wnt4. This study, therefore, indicates that Wnt and Notch signals are functioning together in order to maintain T-cell development throughout the life.

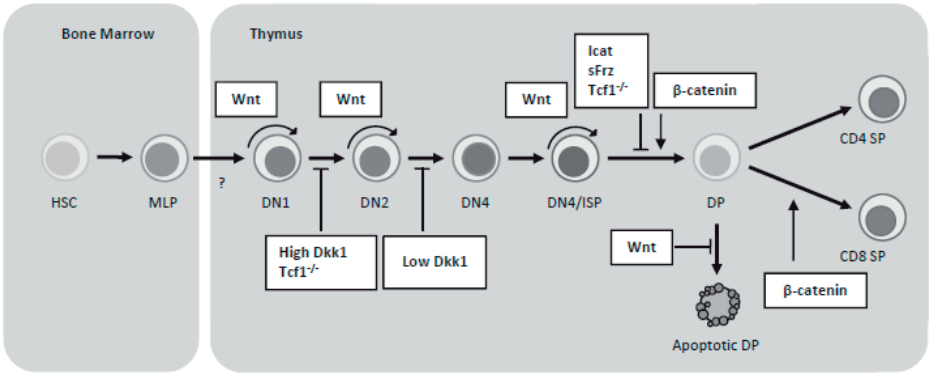

Figure 7. Effect of Wnt signaling during thymopoiesis in the thymus.

The first cells to arrive in the thymus are rare progenitor cells commonly referred to ETPs (early thymic progenitors), which reside in the DN (CD4–CD8– double negative (DN)) compartment. DN cells proliferate rapidly, partly mediated by Wnt signaling. Inhibition of the Wnt pathway, by ectopic expression of soluble Frizzled receptor (which acts as a decoy receptor), Dickkopf homologue 1 (Dkk1; which inhibits binding to Ldl receptor related protein (Lrp) co-receptors) or the cell autonomous inhibitor of β catenin and Tcf (ICAT; which disrupts the β-catenin–Tcf interaction) leads to inhibition of T-cell development at various points in the DN developmental pathway. Similarly, incomplete blocks in T-cell development are observed at DN1, DN2 and ISP (immature single positive) stages of development in Tcf1 (T-cell factor 1)-deficient mice. Wnt signaling also regulates the survival of double positive (DP; CD4+CD8+) thymocytes by upregulating expression of the anti-apoptotic protein Bcl-Xl and stabilized β-catenin effect positive selection and interleukin-7 receptor signaling, resulting in increased numbers of CD8+ SP (single positive) thymocytes. Moreover, the levels of CD4 on both DP and CD4+ SP cells are regulated in part by Tcf1 (not shown in the figure). HSC, hematopoietic stem cells; MLP, multi lineage progenitor.

Non-canonical Wnt pathway

Few reports have also shown the effect of non-canonical Wnt signaling during T cell development. The role Wnt-Ca2+ pathway as an example has been investigated in Wnt5a KO mice, or by providing high levels of exogenous Wnt5a [122]. Wnt5a deficient mice die at birth due to severe anatomical abnormalities; as a result, T-cell development was studied in an ex vivo FTOC using thymic lobes obtained from embryonic day 14 (E14). This study proved that Wnt5a is important in the regulation of the αβ-lineage survival at DP stage. In Wnt5a KO FTOC the expression of proapoptotic gene Bax was downregulated, while expression of the antiapoptotic gene Bcl2 was upregulated, resulting in the inhibition of DP thymocytes apoptosis. On the other hand, exogenous Wnt5a augmented the apoptosis of fetal T cell progenitors [122]. It is also shown that Wnt5a triggers Ca2+ signaling by increasing free cytoplasmic Ca2+ to regulate the DP and mature SP thymocytes survival. Moreover, upregulation of non-canonical Wnt Ca2+ pathway inhibited the canonical Wnt signaling by downregulation of β-catenin expression.

Another study which addressed the role of the non-canonical pathway in thymopoiesis relates to the Wnt4 KO mice that die due to the renal failure shortly after birth [150]. It has shown that lymph-node-resident hematolymphoid progenitors are able to generate mature T cells when they are cultured in the presence but not in the absence of Wnt4 protein. Using Wnt4 KO neonates, Louis et al found that Wnt4 is crucial for the maintenance of the bone-marrow HSCs and to sustain a normal thymic cellularity. In adult murine, Wnt4 overexpression induced the expansion of non-renewing lymphoid-primed multipotent progenitors (LMPPs; Flt3+ LSKs) downstream of hematopoietic stem cells (HSCs; Flt3– LSKs). Expansion of LMPPs was associated with a proportionate accumulation of ETPs and thymic seeding cells resulting in an increase in thymic cellularity. Notably, they showed that Wnt4 induced non-canonical JNK-dependent Wnt pathway [169, 170]. In follow up studies they showed that Wnt4 regulates homeostatic thymic cellularity in a thymic epithelial cell (TEC)–dependent manner. The absence of Wnt4 inhibited fetal and postnatal thymic expansion which led to diminished TEC numbers, a modification of the medullary-to-cortical TEC ratio, and a disproportionate loss of the most immature thymocyte precursors which are highly express c-kit marker. Wnt4 and its downstream signaling pathways could be utilized as a promising candidate to improve thymic cellularity in the process of thymic atrophy [171].

Transcriptional drivers of T cell commitment

T-cell commitment depends on a collection of various transcription factors, each with its own expression profile including Myb, Runx1 with its partner CBFb, GATA-3, TCF-1 (encoded by Tcf7) and Bcl11b and E2A (which encodes two alternative splice variants E12 and E47). Weber et al have shown that TCF-1 is highly expressed in the earliest thymic progenitors, and its expression is upregulated by Notch signals. However, when TCF-1 has ectopically expressed in bone marrow (BM) progenitors, it induces the development of T-lineage cells in the absence of Notch1 signals. Further characterization of these TCF-1-induced cells showed expression of several T-lineage genes, including T-cell-specific transcription factors Gata3 and Bcl11b, and components of the T-cell receptor [172]. In a related study by Germar et al, it has been proven that Tcf-1 is required at the earliest phase of T-cell development for progression beyond the early thymic progenitor stage. The earliest deficiency detected in Tcf-1 KO thymocytes was the reduced expression of c-kit at the DN1 stage of development. Tcf-1–KO cells at this stage showed increased apoptosis and have significantly reduced expression of genes involved in DNA metabolic processes, chromatin modification, and response to damage compared with their WT counterparts [173].

Induction of the T-cell developmental program initially depends on Notch signaling. Notch signaling is required to set up the first T cell specific genes, initially Tcf7, later Gata3 and also Bcl11b, the transcription factor that seals off the T cell commitment fate [174]. However, this signal is not a constant component of the regulatory state mix. Notch responsiveness is indispensable at the DP stage, but once triggered at the early stages of T cell development, most of the regulatory genes that contribute positively to the T-cell program sustain maximal levels of expression even when Notch signaling is not active anymore [175]. Developing T cells initially express a considerable level of PU.1, but inhibit it completely during lineage commitment. This process takes surprisingly long, almost 14 days (at least 10-12 cell divisions) in the mouse and probably longer in humans based on OP9 cultures with human CD34+ cells [176]. Thus, these cells have only a short opportunity in which myeloid potential poses a threat to T lineage fidelity. In fact, the thymic microenvironment is nonpermissive for expression of this myeloid potential [177], although both in mouse and human systems B and myeloid potential can be detected.

The T-cell program is orchestrated by inducing expression of three transcription factors: Gata3, Tcf7, and Bcl11b, These transcription factors rely all in part on Notch signaling via RBP-j. GATA-3 is essential for T-cell development from the earliest stage throughout multiple later developmental checkpoints, and it is restricted in its hematopoietic expression to T cells and T-cell-like innate lymphoid cells [178]. GATA-3 can antagonize alternative lineage fates, through its ability to repress PU.1 and its ability to repress the B-cell program by downregulating Pax5 [179]. In early stages of T cells development GATA-3 expression is not controlled via a positive autoregulation process as it has shown that exogenous GATA-3 is not able to increase endogenous Gata3 expression, and early T cell progenitors lacking Gata-3 gene continue to express RNA from the Gata3 promoter at a comparable levels to the WT counterpart [179]. Thus, It seems that GATA-3 expression level depends on other factors. At the later stages of T-cell development, GATA-3 expression levels are regulated by the signaling pathways responding to TCR engagement and Stat6. Instead, Gata3 control after initial Notch-dependent induction is probably maintained by Myb and TCF-1 as likely positive regulators [180].

T cells commitment completes when thymocytes progress to DN2A stage and then increase expression of the Bcl11b gene [181, 182]. Indeed Bcl11b is turned on by Notch signaling and probably other factors and inhibits residual NK cell lineage potential in DN2 cells [177, 182]. Bcl11b gene expression has one of the most significant increases in the level of expression from the ETP stage to the newly committed DN2b stage [181, 182]. Single-cell analysis using fluorescent reporter for the Bcl11b locus together with reporters for Bcl11a or Spi1 (PU.1) confirmed that ETP cells first activating Bcl11b expression but still sustain the expression of Bcl11a and PU.1, though they become downregulated afterward. DN2b

cells, which are functionally committed T cells, still clearly retain PU.1 protein as shown by single-cell fluorescent staining [183]. Recently the laboratory of Prof. Rothenberg unraveled the mechanisms of Bcl11b activation during T lineage commitment by generating a knock-in fluorescent reporter at the Bcl11b locus and followed Bcl11b activation dynamics at the single-cell level using in vitro developmental assays together with flow cytometry and time-lapse live imaging. They showed that factors that are controlling Bcl11b expression amplitude differ from those that license the locus for expression competence, a regulatory strategy that enables the latter to have subsequent roles in mature T cell functional specialization. These factors work via three distinct, asynchronous mechanisms: an early locus 'poising' function dependent on TCF-1 and GATA-3, a stochastic-permissively function dependent on Notch signaling, and a separate amplitude control function dependent on Runx1, which is already expressed at HSC level [184].

Another transcription factor that is involved in inducing rearrangements in both T and B cells is E2A [185, 186]. Loss of E2A activity results in a partial block at the earliest stage of T-lineage development [187]. This early T-cell phenotype precedes the development T cell leukemias [188], as also occurs in thymocytes lacking Tcf1 [189]. Thus, both E2a and Tcf1 are not only crucial as positively acting transcription factors, but also as tumor suppressor genes for the development of thymic lymphomas/leukemias.

Wnt signaling in hematological malignancies

In most solid tumors, constitutively active Wnt signaling is a significant contributing or even initiating event for the development of such cancers [102]. The prototypical example is colon carcinoma, in which carcinogenesis is caused by inactivating mutations in the tumor suppressors Apc or Axin1 or activating mutations in β-catenin [190]. Recently, it has been shown that deregulated Wnt signaling is also crucial during the development of hematological malignancies. Although the underlying mechanism is not completely clear, mutations leading to the overexpression of Wnt genes or β-catenin and γ-catenin seem to be important.

Acute Myeloid Leukemia (AML)

AML is a clonal malignancy that arises in HSCs or myeloid progenitors cells. AML is frequently associated with chromosomal translocations leading to abnormal fusion proteins (such as AML1–ETO, PML–RARα, PLZF–RARα and CBF1–MYH11) or with activating mutations in the receptor tyrosine kinase Flt3, which is the receptor for the cytokine Flt3L.

Target genes of these fusion proteins have been shown to be associated with Wnt signaling, in particular, γ-catenin [191]. Furthermore, high levels of β-catenin expression in AML cells

exhibit poor prognosis [192]. Eventually, to underscore the significance of Wnt signaling in myeloid cells, myeloid progenitor cells of patients with severe congenital neutropenia had a severe downregulation of Lef1 expression and its target genes. Thus, Lef1 plays an important role in normal human myelopoiesis [193].

Many studies demonstrate that epigenetic inactivation of Wnt pathway inhibitors by CpG island methylation provides an additional mechanism for the observed Wnt-pathway activity in AML leukemic cells. The methylation status of Wnt antagonists, such as sFRP-1, 3, 4, and DKK1, was shown to be responsible for the activation of the Wnt pathway in AML cells and correlated with poor prognosis [194-199].

Another natural antagonist of the canonical Wnt-pathway is the Wnt protein Wnt5a. This particular Wnt protein activates the non-canonical Wnt-pathway and mice hemizygous for Wnt5a develop myeloid leukemias[121]. Also in human samples, Wnt5a appeared to function as a tumor suppressor. In normal B cells, myeloid cells and CD34+ bone marrow cells Wnt5a transcripts were readily detectable. Analysis of several acute lymphoblastic leukemias (ALLs) showed that in samples of both B-ALL and AML the levels of Wnt5a were greatly reduced or completely absent. In conclusion, active Wnt signaling appears to play an important role in the propagation/acceleration of AML and has been shown to be an important secondary oncogenic event in mouse models of AML to transform pre-LSCs into LSCs. Based on these insights, new therapeutic opportunities have been explored by investigators who show that small-molecule Wnt-pathway inhibitors, which inhibit the interaction between β-catenin and LEF1, selectively induce cell death in AML cell lines and primary AML blasts [200]. Recent studies in preclinical settings indicate the promise of Wnt inhibition to treat AML [201-203], suggesting that targeted therapy of leukemia stem cells in AML may become possible.

Chronic Myeloid Leukaemia (CML)

Most of the CML cases carry the classical Philadelphia chromosome, which is caused by a t(9,22) translocation leading to the generation of the abnormal BCR-ABL fusion protein. It has been shown that Wnt signaling is upregulated at the terminal phase of CML in which the disease resembles acute leukemia [204], but it is normal at other stages.

Although β-catenin deficiency in mice significantly prevent the occurrence of BCR-ABL-induced CML, the development of BCR-ABL-induced BCP-ALL was not altered. As CML and ALL might originate from different cells, these studies conclude that the use of Wnt signaling might depend on the tumor origin. In conclusion, as the fusion protein Bcr-Abl can actively modulate β-catenin levels in the cells, the most severely affected Wnt-pathway in CML is the canonical Wnt pathway. However, as recent studies show, in CML cells resistant

to tyrosine kinase inhibitors the non-canonical Wnt-pathway might interfere when the Bcr-Abl-mediated mechanism is inhibited. Therefore, novel therapeutics should not only be aimed at affecting the canonical Wnt-pathway but should also take into account the redundant effects of the non-canonical pathway, all in combination with tyrosine kinase inhibitors targeting BCR-ABL.

Acute Lymphoblastic Leukemia (ALL)

As Wnt signaling together with Notch signals are crucial for normal T-cell development in the thymus, constitutively active Wnt signaling should lead to ALL [205]. Experimental evidence for this hypothesis has been demonstrated by taking advantage of conditional deletion of exon 3 in β-catenin, which results in a constitutively active form of β-catenin. As a result, β-catenin can no longer be phosphorylated and broken down in the proteasome [163]. This causes aggressive T-cell lymphomas that metastasize to the bone marrow and are transplantable into irradiated recipient mice. These tumors arise independently from Notch signals, suggesting that although the Notch and Wnt pathways cooperate during the early stages of T-cell development in the thymus, they can act independently during leukemogenesis. Another study revealed yet another mechanism by which oncogenic forms of β-catenin can cause thymic lymphomas; constitutively active forms of β-catenin were shown to cause p53-independent oncogene-induced-senescence, growth arrest and finally lymphoma development [206].

Of special interest, there are two recent studies that show a clear tumor suppressor role for Tcf1 in T-ALL development [189, 207]. Both studies show that mice deficient for Tcf1 are highly susceptible to develop leukemias. The observed leukemias had a heterogeneous pattern of leukemia formation, which is expected as Tcf-deficiency leads to several incomplete and consecutive T cell blocks in development. Remarkable was the high expression of Lef1 in these leukemias (and Id2 in the DN3 lymphomas in the study by Yu et al). Both studies show that Tcf1 normally acts a suppressor of Lef1 protein levels in the thymus. Upon deletion of Tcf1, Lef1 protein levels become deregulated in all developmentally blocked thymic subsets, resulting in abnormally high levels of the long isoform of Lef1 predisposing the thymocytes towards leukemic transformation. The question remains whether this Lef-mediated oncogenic effect, due to the absence of Tcf1 is a Wnt-mediated driven process or not. The study by Tiemessen et al could demonstrate elevated Wnt signaling activity in the Tcf1-deficient tumors by crossing the Tcf1-deficient mice to a Wnt-reporter mouse. As the study by Yu et al. could not demonstrate this, there might be a possibility that high Lef1 levels mediate leukemia formation in an additional Wnt-independent way. The crossing of the Tcf1-deficient mouse to an inducible Lef1-knockout mouse confirmed the redundancy of both factors as there was an almost complete total lack of T cell development. However, in the double knock-out (Tcf/Lef) mice still, some

leukemia formation (2 out of 13 mice) was reported. This may suggest the existence of an additional Wnt-pathway-independent mechanism causing these lymphomas. However, a key role for Lef1 was also found in a mouse model that deregulates the Notch pathway by ectopic expression of the intracellular domain of Notch1 [208]. This study showed that T cell lymphoma lines require high levels of Lef1 for their survival. Hence, deregulation of Lef1 expression, either via lack of the tumor suppressor Tcf1 or via increasing of the Notch pathway accelerates lymphomagenesis. Also in human cases of precursor T-ALL aberrantly active Wnt signaling has been reported [76, 209].

Aim of the thesis

The capability of HSCs to self-renew and differentiate towards T cells is precisely controlled by multiple signals including Wnt signaling, supplying from the niche within the BM and thymus. Therefore, exploring the exact micro environmental signals that support HSCs and T cell progenitors development has been a major goal. This thesis describes studies aimed to understand the role of Wnt signaling pathways involved in the regulation of HSC self-renewal and T cell development in the BM and thymus respectively. Better understanding the role of Wnt signaling in hematopoiesis and thymopoiesis could be clinically relevant to improve delayed T cell reconstitution after SCT either by applying during ex vivo culture systems of HSCs before transplantation or by administrating in vivo to boost and/or sustain T cell potential. Therefore, the main aim of the studies in this thesis was to unravel the functional role of both canonical and non-canonical Wnt signals for hematopoiesis and T cell development.

As most of the studies on the role of Wnt signaling in hematopoiesis and T cell development have focused more on the canonical Wnt signaling, in this thesis we gave an equal attention to both canonical and non-canonical Wnt in hematopoiesis and lymphopoiesis. Chapter 2 is a follow-up study on the report of Luis et al. in which our group showed that high level of Wnt signaling impaired HSC repopulation. Using gene expression analysis as well as functional studies, we explained the responsible mechanism underlying this observation. In chapter 3 we studied the role of a noncanonical Wnt receptor Ryk in hematopoiesis and lymphopoiesis. We used a loss of function model of Ryk KO mouse and performed in vitro and in vivo reconstitution assays. Chapter 4 of the thesis examines effects of both canonical and non-canonical Wnt signaling side by side during lymphopoiesis. In order to do that we used two gain of function models namely Wnt3 and Wnt5a overexpression in in vitro and in vivo differentiation assays. Chapters 5 and 6 describe the role of Tcf-1 in T cell development and malignancy using a Tcf-1 deficient mouse model. Chapter 5 studied the role of this transcription factor in a loss-of function model to discover the role of Tcf-1 in T cell commitment. Chapter 6 explains the role of Tcf-1 in thymic lymphoma

development. Lastly, Chapter 7 discusses the significance and implications of the studies described, and provide directions for future research.

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CHAPTER 2

High Levels of Canonical Wnt Signaling Lead to Loss of Stemness and Increased Differentiation in Hematopoietic Stem Cells

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Summary

Canonical Wnt signaling regulates the self-renewal of most if not all stem cell systems. In the blood system, the role of Wnt signaling has been the subject of much debate but there is consensus that high Wnt signals lead to loss of reconstituting capacity. To better understand this phenomenon, we have taken advantage of a series of hypomorphic mutant *Apc* alleles resulting in a broad range of Wnt dosages in hematopoietic stem cells (HSCs) and performed whole-genome gene expression analyses. Gene expression profiling and functional studies show that HSCs with APC mutations lead to high Wnt levels, enhanced differentiation, and diminished proliferation but have no effect on apoptosis, collectively leading to loss of stemness. Thus, we provide mechanistic insight into the role of APC mutations and Wnt signaling in HSC biology. As Wnt signals are explored in various in vivo and ex vivo expansion protocols for HSCs, our findings also have clinical ramifications.

Introduction

In many tissues, including the blood, intestine and skin, old cells are eliminated and replenished by newly developed cells from a small pool of stem cells. This rare population of stem cells is located in a specific microenvironment, the niche, and gives rise to several different lineages of abundant daughter cells (Mendez-Ferrer et al., 2010). The signals controlling the various stem cell fates (self-renewal, differentiation, quiescence, apoptosis, and others) are beginning to be elucidated. A number of evolutionary conserved pathways are important for the development and maintenance of adult stem cells, including Notch, bone morphogenic protein, hedgehog, fibroblast growth factor, transforming growth factor- β, and Wnt signals (Blank et al., 2008). Among these pathways, the Wnt pathway is seen as a dominant factor in self-renewal of many types of adult stem cells (Reya and Clevers, 2005). Compared with the convincing studies on the role of Wnt signaling in adult stem cells in skin and gut, a role for Wnt in adult hematopoietic stem cells (HSCs) has proved much more difficult to demonstrate (reviewed in Luis et al., 2012). In studies reporting an important role for Wnt signaling in blood cells, Wnt seemed to be required for normal HSC self-renewal and therefore for efficient reconstitution after transplantation (Luis et al., 2011).

Several types of Wnt signaling can be discerned often referred to as the canonical or Wnt/β-catenin pathway and the non-canonical pathways (reviewed extensively in Staal et al., 2008). In the absence of Wnt ligands, cytoplasmic levels of β-catenin are kept very low through the action of a protein complex (the so-called destruction complex) that actively targets β-catenin for degradation. This complex is composed of two negative regulatory kinases, including glycogen synthase kinase 3β (GSK-3β) and at least two anchor proteins that also function as tumor suppressor proteins, namely Axin1 or Axin2 and APC (adenomatous polyposis coli). APC and Axin function as negative regulators of the pathway by sequestering β-catenin in the cytoplasm. Hence, inactivating mutations in *Apc* lead to higher β-catenin protein accumulation among other important events controlled by APC. Activation of the pathway by Wnt leads to inactivation of the destruction complex allowing buildup of β-catenin and its migration to the nucleus. In the nucleus, β-catenin binds to members of the TCF/LEF transcription factor family, thereby converting them from transcriptional repressors into transcriptional activators.

Initial attempts to overexpress a constitutively active form of β-catenin in HSCs led to an increase in proliferation and repopulation capacity upon transplantation into lethally irradiated mice (Reya et al., 2003). However, later studies using conditional overexpression of a stabilized form of β-catenin led to a block in multilineage differentiation, and the exhaustion of long-term HSCs (Kirstetter et al., 2006; Scheller et al., 2006). This resulted in anemic mice and eventually led to lethality, i.e., the opposite effect when compared with

the improved transplantation setting reported earlier. These studies have created confusion concerning the importance of Wnt in maintaining numbers and integrity of HSCs. Similarly, not all loss-of-function studies have produced clear phenotypes. The Mx-Cre system has been used to drive deletion of β-catenin (Zhao et al., 2007) or both β-catenin and its homolog γ-catenin (Koch et al., 2008; Jeannet et al., 2008). However, no defects were reported in HSC function or cells within lymphoid tissues. Surprisingly, in vivo reporter assays revealed that the canonical Wnt signaling pathway was still active in HSCs despite the absence of both β- and γ-catenin (Jeannet et al., 2008). This could imply the existence of an alternative factor or generation of a hypomorphic allele permitting low levels of Wnt signaling that would negate hematopoietic defects. Heroic efforts to knock out the *Porcn* gene during hematopoiesis, which encodes an acyltransferase (porcupine) necessary for acylation of Wnts, enabling their secretion and binding to the frizzled receptors, have not resulted in hematopoietic defects; however, there also were no changes in Wnt signaling (Kabiri et al., 2015). The reasons for this are presently unknown, but incomplete deletion or the lack of need for Wnt secretion have been suggested (Oostendorp, 2015). This demonstrates the high complexity and difficulty in generating bona fide null mutants for canonical Wnts in the hematopoietic system. Together with studies in which Wnt activity in HSCs was reported to be close to zero (Fleming et al., 2008; Luis et al., 2009; Zhao et al., 2007), these findings suggest that complete absence of Wnt signaling is detrimental to HSC function, but that up to a quarter of normal activity is sufficient for normal function. Our recent findings suggest that these very different results in both gain- of-function and loss-of-function studies can be largely explained by differences in levels of Wnt signaling achieved in different experimental circumstances. That is, when Wnt signaling is slightly enhanced over normal levels, HSCs show improved reconstitution capacity. However, when HSCs express high levels of Wnt signaling, they completely fail to reconstitute irradiated recipient mice (Luis et al., 2011). Thus, different levels of activation of the pathway can account for the discrepancies in previous studies (Malhotra and Kincade, 2009).

Results

Gene Expression Profiling and Correlation with Wnt Dosage

Previously, we have used a combination of two different hypomorphic alleles and a conditional deletion allele of the *Apc* gene resulting in a gradient of five distinct levels of Wnt signaling in vivo. In the *Apc*1572T and *Apc*1638N alleles, amino acid residues 1572 and 1638 have been targeted resulting in different levels and lengths of truncated Apc proteins, consequently leading to different levels of Wnt pathway activation. Deletion of *Apc* exon 15 within the Apc^{15lox} allele was performed ex vivo by using a Cre-recombinase encoding retrovirus (Figure 1A). LSK cells from wild-type (WT) mice ($Apc^{+/+}$) transduced with the same viral construct were employed as controls for all experiments. Transduced cells were sorted and employed for gene expression profiling by Affymetrix genome-wide microarrays. In the current report, we focused on the differences between WT LSK cells, which efficiently reconstitute recipient mice, and the LSK cells with increased Wnt signaling activity (*Apc*1572T, *Apc*1638N, and the Apc^{15lox} mutant alleles). Biological triplicates were used for each condition. As WT HSCs have low but detectable and slightly variable levels of Wnt signaling, and they form the basis for comparison of all other conditions, we used six replicates for WT HSCs.

Principal component analysis showed clear separation of the triplicate arrays per genotype corresponding to the different Wnt signaling levels (Figure 1B). Hierarchical clustering of the top 50 differentially expressed genes also revealed a clear separation of the different Wnt signaling clusters (Figure 1C).

Biological Processes Correlated with High Wnt Levels in HSC

Focusing on the most differentially expressed genes, a heat-map was constructed that clearly reveals the differences between WT and Apc^{15lox} HSCs (Figure 2A). We used the gene expression data of all available probe sets across the 15 APC samples and applied Barnes-Hut t-distributed stochastic neighbor embedding (t-SNE) to map each individual gene or probe set into a 2D space. The 2D landscape illustrates genes/probe sets with similar behavior (Figure 2B). Genes that have highly correlated expression profiles will be located in close proximity in the map, whereas uncorrelated expression profiles should be far apart in the t-SNE map. Genes that follow the increase in Wnt signaling cluster in a set of genes composed of known Wnt target genes, such as Axin2, Tcf7, and Lef1 (Figures 2C–2F). Genes that are anti-correlated with increased Wnt signaling can also be discerned and include *Ccr9* and *Cd3g* (Figures 2G–2I).

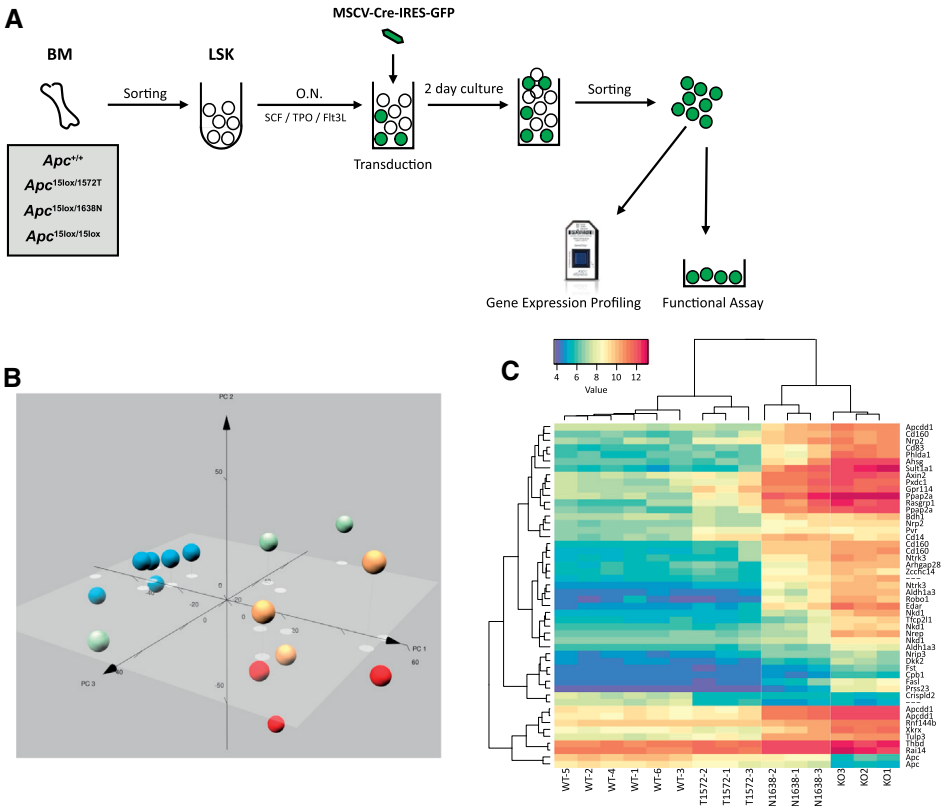

Figure 1. Definition of a High Wnt Stem Cell Signature.

(A) Experimental setup. LSK cells from various APC mutant mice were sorted from bone marrow, transduced with Cre-GFP retrovirus and GFP-transduced cells were again sorted and used for further experiments. (B) Principal component analysis plots of all 15 biological samples used in this study. The percentage of variance captured by each of the first three principal components is indicated. (C) Hierarchical clustering of the various APC mutants and WT HSCs indicating the top 50 differentially expressed genes and changes in gene expression.

The differential gene expression as detected by microarray analysis was validated using digital Q-PCR (Figure S1A). Checking the biological processes involved in the differences between low and high Wnt signaling, we observed gene sets found in Wnt and Notch signaling but also differentiation into monocytes, myeloid cells, and B lymphocytes (Figure S1B). No differences were observed in apoptosis or cell-cycle-related genes. We confirmed these findings by specifically selecting published gene sets for these processes and checking whether clustering with the published gene sets correlated with the Apc mutants. The differentially expressed genes we found were highly enriched in the B lymphoid and myeloid differentiation sig-natures but not for pro-apoptotic or anti-apoptotic genes (Figures S1B, S2, and S3).

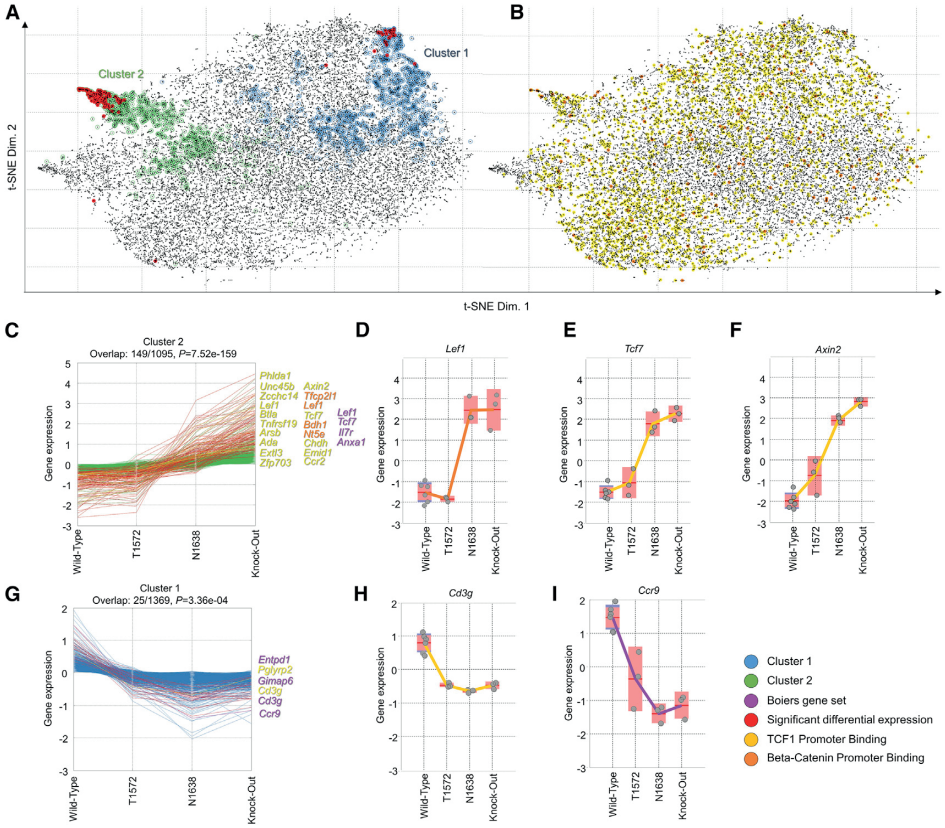

Figure 2. t-SNE Landscape of APC Mutants.

(A and B) t-SNE maps of all probe sets. Red colored lines are differentially expressed genes, green are in cluster 15, yellow show both binding (TCF1/TCF7 or β-catenin), and differential expression. Text labels are shown only for the latter. (C and G) Cluster 2 and 1 identified in t-SNE. (D–F, H, and I) Selected genes with their expression in the various Apc mutants.

Apc Mutants Causing High Levels of Wnt Signaling Inhibit Proliferation but Do Not Change Apoptosis

Ming et al. (2012) reported that HSCs with high Wnt signals have increased apoptosis due to a high level of Wnt signaling and impaired self-renewal in HSCs. In their study, an activated form of β-catenin was used resulting in increased Wnt signaling in HSCs to the same level as the *Apc*1638N mutant used here. We therefore also used a constitutively active β-catenin conditional allele targeted the same way as the conditional 15lox APC $^{-/-}$ LSK cells to check the *Axin2* levels as readout for the Wnt signaling dosage. The β-catenin (ΔEx3) allele (Harada et al., 1999) gave 21-fold higher *Axin2* levels in LSK cells compared with WT LSK cells transduced with GFP-Cre, whereas the 1638N resulted in 23-fold and the Apc15lox ~50-fold higher *Axin2* mRNA levels. Thus, the *Axin2* levels and hence activation of the Wnt pathway

were similar. However, our gene expression analysis did not show any significant differentially expressed genes associated with apoptosis. In order to study the putative involvement of apoptosis with a more functional approach, we performed two different apoptosis assays. First, we assessed apoptosis by annexin V/7-amino-actinomycin (7-AAD) staining of the ex vivo transduced LSK cells from Apc WT and $Apc^{15lox/15lox}$ (Figure 3A). At the beginning of culture, there was almost no apoptosis in both groups (~4% at day 0). After 3 days of culture, the percentage of annexin V^+ apoptotic cells increased to ~16%. However, no difference was observed between the Apc WT and knockout (KO) groups. Next, we performed caspase-3 staining in order to assess the apoptosis rate of ex vivo transduced LSK cells (Figure 3B). Similar to previous assays, there was hardly any caspase-3 positivity at the beginning of the culture, while it was elevated after 3 days of culture. However, again no difference was observed between the two groups. Subsequently, we analyzed the proliferation status of the transduced LSK cells by labeling the cells with proliferation dye EF670 (Figure 3C). While cells did not proliferate at the beginning of culture (filled gray histogram), *Apc* WT LSK cells proliferated around 4-fold more than *Apc* KO LSK cells. Therefore, although a high level of Wnt signaling does not affect apoptosis, it decreases proliferation of LSK cells after 3 days of culture.

High Wnt HSCs Show Enhanced Myeloid and B Lymphoid Differentiation Capacity

Our gene expression analysis revealed that LSK cells with high levels of Wnt induce upregulation of B and myeloid-associated genes (Figure S2). In order to confirm this observation functionally, we performed in vitro B and myeloid differentiation assays using the OP9 stromal cell line (Figure 4). LSK cells were sorted, transduced with the Cre-GFP retrovirus, and cultured for 14 days on OP9 cells. *Apc* lox15 LSK cells developed to granulocytes ($CD11b^+$ $Gr1^+$) with around 2-fold higher frequency, and developed to B cell line-age ($B220^+$ $CD19^+$) with around 2.5-fold higher frequency compared with WT LSK cells. Thus, we confirmed by functional assays that Apc mutations leading to a high level of Wnt signaling enhance differentiation toward B and myeloid lineages.

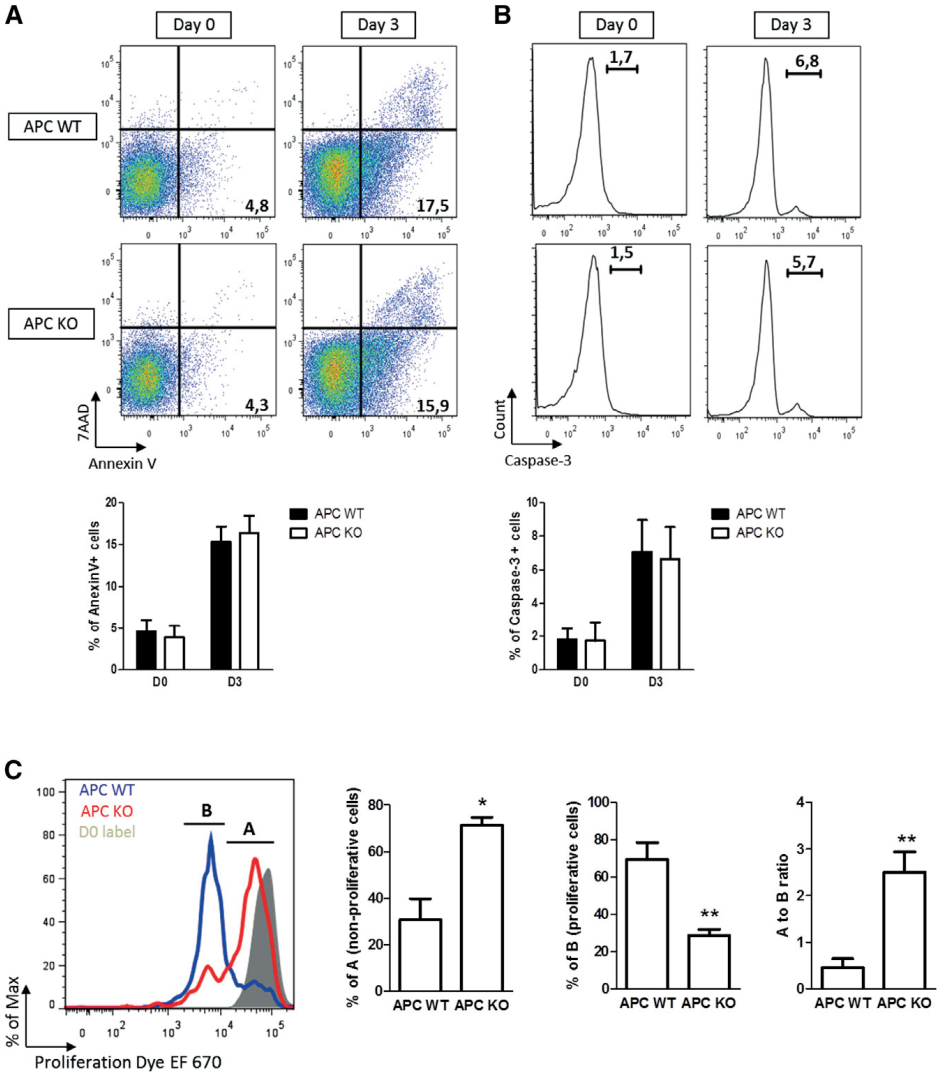

Figure 3. High Levels of Wnt Signaling Do Not Affect Apoptosis.

*(A and B) Sorted BM LSK from Apc WT and 15lox/15lox were transduced with Cre virus and cultured for 2 days to fulfill Cre recombination activity. After culturing for 2 days (day 0) and 5 days (day 3), cells were harvested and stained with annexin V/7-AAD (left graph) or active caspase-3 (right graph). Error bars represent the SD of three replicates of one independent experiment. (C) Sorted BM LSK from Apc WT and 15lox/15lox were transduced with Cre virus, cultured for 2 days and labeled with 5 mM proliferation dye EF670 or with DMSO. The left plot depicts representative histogram plots and the right graphs show the percentage of non-proliferative cells (A), proliferative cells (B), and ratio of A/B. Error bars represent the SD of three samples from individual mice in one independent experiment. Two independent experiments were done with similar outcome. *$p < 0.05$ and **$p < 0.01$ (Mann-Whitney U test).*

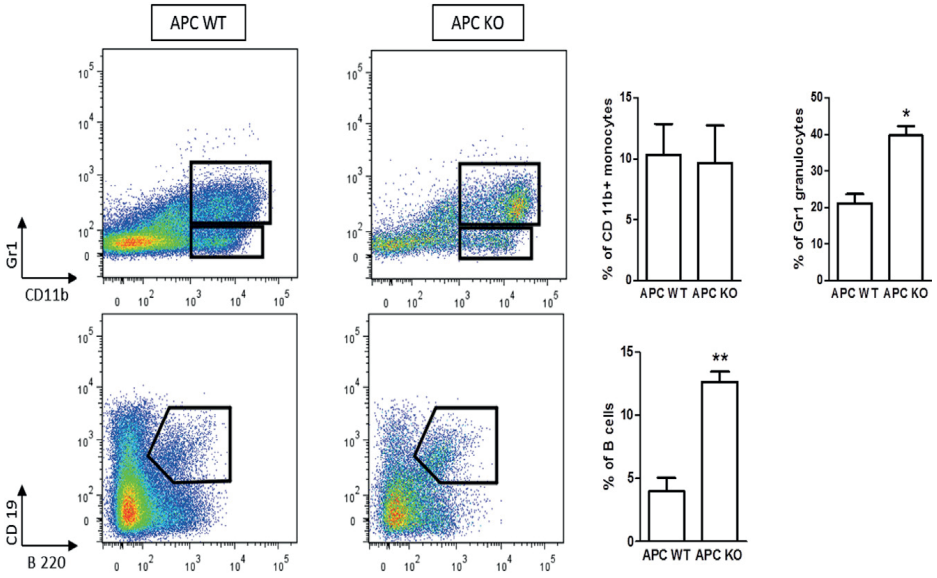

Figure 4. High Levels of Wnt Signaling Enhances Multilineage Differentiation.

*Transduced LSK cells from Apc WT and 15lox/ 15lox were co-cultured with OP9 stromal cell line for 14 days, then were harvested, and assessed by flow cytometry for myeloid (CD11b and Gr1+) and B cell development (B220 and CD19+). Error bars represent the SD of six samples from individual mice from two independent experiments. Asterisks indicate statistical significance as follows: *$p < 0.05$, and **$p < 0.01$ (Mann-Whitney U test).*

Discussion

The Wnt signaling pathway has emerged as the dominant self-renewal pathway for various adult-type stem cells and is required for maintenance of embryonic as well as induced pluripotent stem cells. In the hematopoietic system, only mild increased Wnt dosages result in higher stem cell activity; indeed the overall Wnt signaling levels in HSC are much lower than those found in intestinal, skin, or mammary gland stem cells. Nevertheless, complete loss of Wnt signaling leads to defective self-renewal as shown in secondary transplantations. This had led to interest in the use of Wnt signaling or factors that modulate Wnt signaling, such as prostaglandin E2 (PGE2) (Goessling et al., 2009) or GSK-3b inhibitors (Huang et al., 2012), for expansion of HSCs ex vivo.

We previously demonstrated that Wnt signaling functions in a strictly controlled dosage-dependent fashion (Luis et al., 2011). As also shown by several other laboratories (Kirstetter et al., 2006; Ming et al., 2012) (Scheller et al., 2006), high Wnt levels in HSCs eventually lead to stem cell exhaustion and lack of reconstitution of irradiated recipients. In the current study, we used gene expression profiling to understand why *Apc* mutations that lead to high Wnt signaling (among other defects) in HSCs would lead to loss of repopulating capacity. Our results show, both at the genetic level and in functional assays, increased differentiation, diminished proliferation, and no effects on apoptosis. The much stronger differentiation toward mature blood lineages coupled with loss of HSC proliferation (see also Figure S4) is expected to lead to lower reconstitution by HSCs. Collectively, these data explain the lack of maintaining bona fide stemness in *Apc* exon 15 deleted HSCs. Thus, instead of increased apoptosis of HSCs, here we offer another explanation for the loss of reconstitution capacity induced by high Wnt levels.

An alternative interpretation of our data is that the observed consequences of *Apc* mutant alleles are not Wnt but rather APC dependent. *Apc* encodes for a multifunctional protein involved in a broad spectrum of cellular functions (Gaspar and Fodde, 2004). To date, most *Apc* mutant mouse models are characterized by tumor phenotypes that depend completely on Wnt dosage. Apc1638T, the only targeted *Apc* mutation that does not affect Wnt signaling at all, results in homozygous viable and tumor-free animals, notwithstanding the deletion of the C-terminal third of the protein containing many functional domains (Smits et al., 1999, 2000). Deletion of only a few amino acids encompassing crucial Axin-binding motifs results in Wnt signaling activation, tumor formation, and lack of reconstitution by HSCs, as we have shown before (Luis et al., 2011). Finally, mutations affecting other members of the Wnt pathway, such as Gsk3β and β-catenin, result in levels of signaling activation and hematopoietic defects that are fully in agreement with our results (Goes-sling et al., 2009; Huang et al., 2009, 2012; Lane et al., 2010). Therefore, the

most likely explanation is that specific levels of Wnt signaling are the major determinant of the observed differential effects on hematopoiesis. In addition, recent studies using recombinant Wnt3a also showed a dose dependent effect on HSC biology (Famili et al., 2015) where high Wnt3a leads to loss of human HSC proliferation in vitro (Duinhouwer et al., 2015), underscoring the differential effects we also have observed with the different *Apc* alleles and correlating exactly with the Wnt dosages caused by these mutations.

The finding that the Apc 15lox mutant leading to high Wnt signaling levels is associated with increased numbers of differentiated cells is not unprecedented. In the intestine, Wnt signaling induces maturation of Paneth cells that contain active β-catenin and Tcf4 (van Es et al., 2005), confirming that high Wnt signaling levels can drive differentiation processes.

Other investigators have used a different system to increase Wnt signals in HSCs, namely overexpression of an oncogenic, constitutively active form of β-catenin (Ming et al., 2012). They showed an increase in apoptosis using annexin V/propidium iodide staining from 10% in WT LSK cells to 35% in high Wnt LSK cells. The reasons for the differences with our results could be due to differences in the systems used, although both are expected to lead to high Wnt signaling levels. Possibly activated β-catenin also negatively affects cell adhesion and homing properties thereby decreasing exposure to important survival signals leading to increased apoptosis. It is also noteworthy that enhanced survival signals are needed to have HSCs survive in the oncogenic β-catenin system. In addition, Li et al. (2013) have shown that Apc regulates the function of HSCs largely through β-catenin-dependent mechanisms, thus demonstrating that, in both systems, canonical Wnt signaling is the major factor.

Whatever the exact mechanism, it is clear that Wnt signaling levels need to be strictly controlled. It is well possible that somewhat higher Wnt levels, which are detrimental to stemness, can be tolerated if HSC survival is enhanced, which then would lead to better self-renewal at this somewhat higher Wnt signaling dose. For instance PI3K/Akt signaling (Perry et al., 2011), as well as expression of Bcl2 (Reya et al., 2003) can provide such signals. Apparently, high Wnt signaling levels can be tolerated in HSC in combination with activation of other survival pathways. Intriguingly, the high Wnt levels in combination with oncogene activation in acute myeloid leukemia seem to allow the Wnt pathway to function as a self-renewal factor for leukemic stem cells (Wang et al., 2010), whereas high Wnt levels cannot do so in normal HSCs. The different localization of normal versus malignant HSCs in the bone marrow niche (Lane et al., 2011) may also contribute to this differential outcome of high Wnt dosage and opens up a therapeutic window targeting leukemic but not normal stem cells.

Experimental procedures

Mice

Mice were bred and maintained in the animal facilities of Leiden University Medical Center, in accordance with legal regulations in the Netherlands and with the approval of the Dutch animal ethical committee.

Microarray Analysis

In this study, we measured the genome-wide gene expression profiles in 21 APC C57Bl/6 mouse samples using Affymetrix mouse 430 2 microarrays for four different conditions; six APC WT, three APC 15lox/1572T, three APC 15lox/1638N, and three APC 15lox/ 15lox mice. 40,000–70,000 sorted LSK cells were stimulated over-night in serum-free medium (STEMCELL Technologies) supplemented with cytokines and transduced by spinoculation with MSCV-Cre-IRES-GFP. Subsequently, Cre-GFP-expressing LSK cells were isolated using flow cytometric cell sorting and collected for RNA expression. RNA of more than 10,000 cells was amplified and processed using the Encore Biotin module and hybridized to Affymetrix mouse 430 2.0 Genechip arrays. Differential expressed genes were determined using Limma, and genes were considered to be differentially expressed if mRNA levels differ with $p < 0.05$ after multiple test correction using Holm. The dataset associated with this study has been deposited at GEO: GSE79495.

Flow Cytometry

Cells were stained in fluorescence-activated cell sorting buffer at 4 ˚C, washed, and measured either on a Canto I or an Aria (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Proliferation, Apoptosis, and Differentiation Assays

For apoptosis, cells were harvested after 2 days (day 0) or 5 days (day 3) of culture, and stained with either 7-AAD/annexin V (BD Bioscience), or phycoerythrin-active caspase-3 apoptosis kit (BD Pharmingen). For the proliferation assay, cells were labeled with 5 mM Cell Proliferation Dye eFluor 670 (eBioscience) at day 0. Subsequently, cells were harvested at day 3 and were assessed for proliferation. For differentiation assays, LSK cells were transduced at day 0 and transferred onto confluent monolayers of OP9 WT. After 14 days, cells were harvested and assessed by flow cytometry for B and myeloid lineage differentiation.

Acknowledgments

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Supplemental Information

Supplemental Experimental procedures

Mouse bone marrow (BM) cells were isolated from femurs and tibiae, which were crushed in a mortar and filtered through 70 µm filters. The cells were stained using biotinylated lineage antibodies (MAC-1/CD11b, B220/CD45R, CD3e, CD4, NK1.1, Gr1, Ter119), Streptavidin PE, CD117 APC and Sca1 PECy7. LSK cells were isolated using a BD Aria II SORP cell sorter (Beckton-Dickinson) and were collected in Stemspan (Stem Cell Technologies), supplemented with mFlt3L (50 ng/ml), rmSCF (100 ng/ml) and rmTPO (10 ng/ml, all cytokines purchased from R&D sytems. The cells were incubated for 16 hr at 37°C and 5% CO2. LSKs from Apc 15 Lox heterozygous mice with mildly elevated Wnt levels were shown to perform better in reconstitution experiments but are not integral part of the current study, as only subtle changes in gene expression were found.

Retroviral Production and Transduction

MSCV-Cre-IRES-GFP plasmid was kindly provided by H. Nakauchi (Institute of Medical Science, University of Tokyo, Japan) and viruses were generated with the Phoenix-packaging cell line. 40,000–70,000 sorted LSKs were stimulated overnight in serum-free medium (StemCell Technologies) supplemented with cytokines (100 ng/ml rmSCF, 10 ng/ml rmTPO, and 50 ng/ml rmFlt3L; from R&D) and transduced by spinoculation (800 x g, 2 hours, 32°C) with titrated amounts of virus with Retronectin (Takara Bio Inc.). Cells were cultured for 2 additional days. Subsequently, Cre-GFP expressing LSK cells were isolated using flow cytometry cell sorting and collected for RNA expression. For in vitro assays including apoptosis, proliferation and differentiation assays bulk of transduced and un-transduced cells were used.

RNA amplification

RNA was isolated from the sorted transduced cells using Qiagen RNEasy micro columns (Qiagen, Hilden, Germany). RNA of more than 10,000 cells were then amplified using the Ovation RNA amplification system v2(Nugen Inc., San Carlos, CA, USA), processed using the Encore Biotin module (Nugen) and hybridized to Affymetrix mouse 430 2.0 Genechip arrays. Data is available at the NCBI Gene Expression Omnibus (GEO), accession number GSE79495.

Gene expression normalization. Gene expression data was measured in two batches. Raw data is normalized per batch with Robust Multi-Array Average (RMA), and batch correction is applied using Combat. Intensity values were mean centered per probe set. Gene symbols are mapped using MM9. As a result of the normalization, probe-intensity values follow a normal distribution for which intensities higher than 0 are up-regulated, and intensities lower than 0 are down-regulated. Principal component analysis and pairwise

correlations across the 21 samples showed the expected results; wild-type and mutants, t1572, n1638, and Knock-Out samples are different from each other in the PCA-space and correlation map.

Gene expression analysis. Differential expressed genes for the APC samples are determined by using Limma, and genes are considered to be differential expressed between the two selected groups if mRNA levels differ with $P<0.05$ after multiple test correction using Holm.

ChIP-Seq normalization. In this study we used massively parallel sequenced DNA-fragments bound by the transcription factors, TCF1, TCF7, and β-catenin. All the sequencing data is aligned using Burrows-Wheeler transformation (BWA), according MM9. We used several literature sources (Li et al., 2013a; Steinke et al., 2014; Zhang and Li, 2008; Zhang et al., 2000) (Wu et al., 2012).

ChIP-Seq analysis. Binding of transcription factors is determined by utilizing Hypergeometric Analysis of Tilling arrays (HATSEQ). A binding event was called when fragments are enriched based on default parameter settings, i.e., FWER significance level < 0.05, and a bandwidth (fragment size) of 300bp. We mapped the significantly detected binding sites to RefSeq genes in UCSC mm9 database (genome.ucsc.edu). A gene was designated as the target gene if the peak was present within 5000bp upstream of the transcription start site or inside of the gene.

For TCF1 (in mature CD8 T cells, accession number GSM1258235), we detected 591 significantly enriched regions (ranges between 104bp-1048bp, median: 233bp) by comparing it to control IgG using sorted post- select DP and CD4+8lo thymocytes1 (accession number GSM1258236). The detected regions could subsequently be mapped to 116 unique genes. For the two TCF1 experiments in murine thymocytes (GSM1285796 for TCF1-CAT and GSM1133644 for TCF1), we detected 732 (size ranges between 102bp- 2632bp, median: 237bp), and 2600 (102bp-2632bp, median: 237bp) significant binding regions respectively after comparing to control TCF1-CAT-INPUT (GSM1285797) and TCF1-INPUT (GSM1133645) respectively. The detected regions could subsequently be mapped to respectively 131, and 653 unique genes (Table S2). The third analyzed ChIP-Seq data set was the binding of TCF7 (GSM773994). For TCF7 we detected 6395 significant binding regions (size ranges between 103bp-5840bp, median: 341bp) by comparing it to one control (input DNA of TCF7). These regions are subsequently mapped to 2015 genes (Table S2). The fourth public data set that we analyzed were three Beta-Catenin experiments, two with biotinylation and one based on FLAG-tag technology. As a background four different controls are used per experiment (2 with Beta-Catenin biotin without GSK and two GSK input samples). This resulted in

respectively 990, 385, and 671 significant binding regions for Beta-Catenin-Biotin-rep1, Beta-Catenin-Biotin-rep2, and Beta-Catenin- Flag-rep1 and were mapped to 121, 49, and 79 genes (Table S2). Binding sites have median size of 336bp, 385bp, and 320bp.

To test the validity of the detected binding regions of each experiment, we expected an overrepresentation of WNT-associated genes. To test this, we overlaid the mapped genes with known WNT-associated genes (n=1136) from the Molecular Signature Database (MSigDB, v4.0), and detected that all seven ChIP-seq experiment showed a significant enrichment for binding in close vicinity of WNT-associated genes ($P<0.05$, Table S1) based on the hypergeometric test. As an example, all seven experiments showed binding of in the transcriptional start site of *Axin2* (Figure S4A), whereas TCF1, and Beta-Catenin experiments showed also binding for *Lef1* (Figure S4A).

Pathway Analysis. Pathway analysis is performed by utilizing the Molecular Signature Database (MSigDB, v4.0) for the detection of enriched curated gene sets (C2), motif gene sets (C3), computational gene sets (C4), GO gene sets (C5), oncogenic signatures (C6), and immunologic signatures (C7). Gene sets and signatures are considered statistically significant when the P-value, derived from the hypergeometric test, is less or equal than 0.05 after correcting for multiple testing using Holm.

Mice

Mice were bred and maintained in the animal facilities of Leiden University Medical Center, in accordance with legal regulations in The Netherlands and with the approval of the Dutch animal ethical committee. C57Bl/6-CD45.1 (Ly5.1) and C57Bl/6-CD45.2 (Ly5.2) mice were obtained from the Jackson Laboratory. Mice carrying targeted mutations on Apc were previously described (Fodde et al., 1994; Robanus-Maandag et al., 2010; Smits et al., 1999) and continuously backcrossed to C57Bl/6 background.

Flow Cytometry

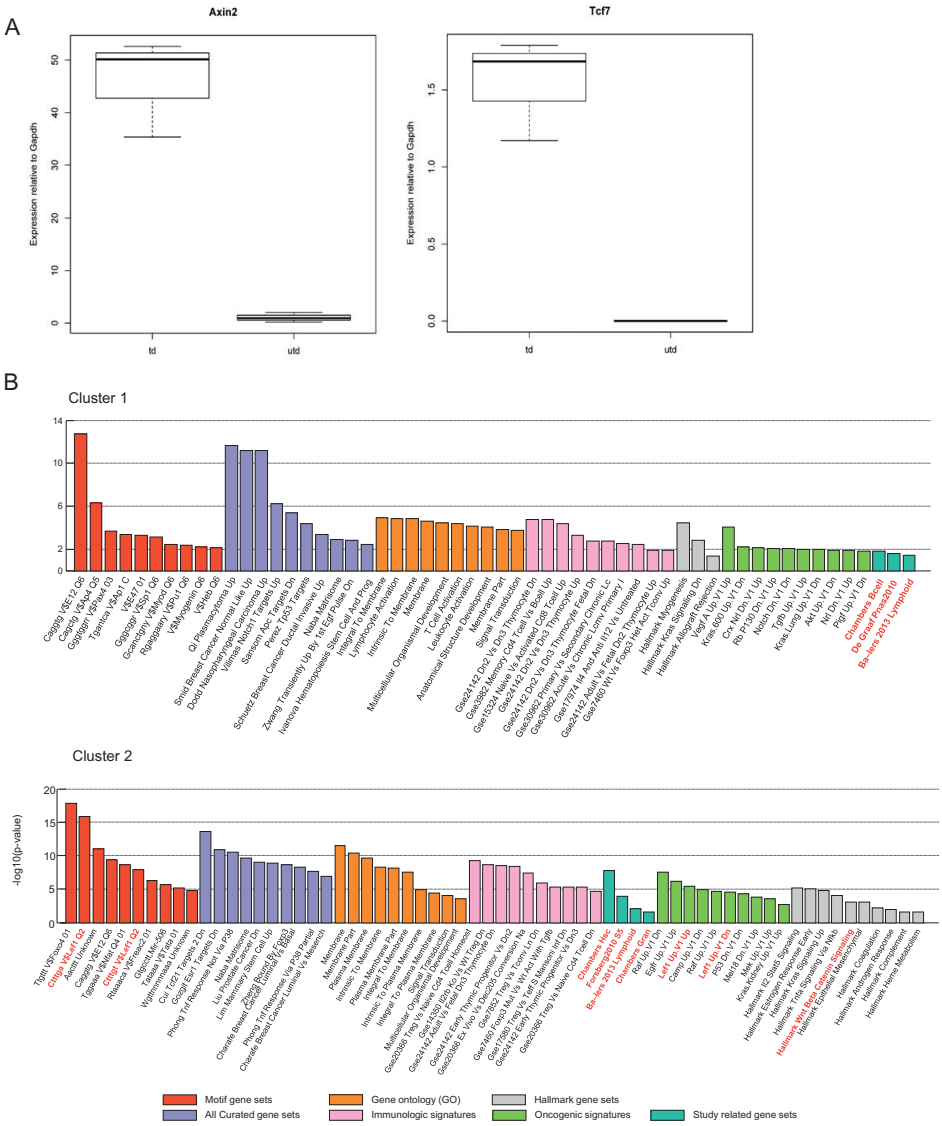
The following antibodies were obtained from BD Biosciences (San Diego, CA): anti CD11b-PE (M1/70), anti CD19-APC (ID3) and anti CD117 (2B6). For Lineage depletion these markers were used: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), Gr1 (RB6-8C5), B220 (Ra3-6B2), Ter119 (Ly76) and Nk1.1 (PK136) biotin and subsequently were stained with streptavidin eFluor 450 (48-4317) from eBioscience. The following antibodies were also purchased from eBiosiences: B220 PE-Cy7 (RA3-6B2), Gr1 eFluor 450 (RB6- 8C5) and Sca1 PE-Cy7 (D7). Cells were stained in Fluorescence activated cell sorter (FACS) buffer (PBS, 2% bovine serum albumin, 0.1% sodium azide) for 30 min at 4 °C. Ultimately, Cells were washed and measured either on a Canto I, or an Aria (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Proliferation, apoptosis and differentiation assays

5 × 104 sorted BM LSKs from APC WT and APC 15lox/15lox mice were transduced with titrated amount of CRE viruses in stemspan with FTS cytokines as previously described. For apoptosis assay harvested cells after 2 days (Day 0) or 5 days (Day 3) of culture, cells were stained with either 7AAD/AnnexinV (BD Bioscience), or PE-Active caspase-3 apoptosis kit (BD pharmingen) according to the manufacturer's instruction. For proliferation assay, cells were labelled with 5 uM Cell Proliferation Dye eFluor® 670 (eBioscience) at Day 0. Subsequently, cells were harvested at Day 3 and were assessed by flow cytometry for proliferation.

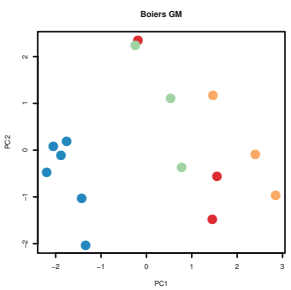
For differentiation assay 2 × 104 BM LSKs were used and transduced cells at Day 0 were transferred onto confluent monolayers of OP9 WT and cocultured for additional 14 days with AlphaMEM 10% FCS containing 50 ng/ml rmSCF, 10 ng/ml rmFlt3L and 10 ng/ml rmIL-7 (all cytokines from R&D). After 7 days cells were harvested and transferred onto new monolayer of OP9 cells, and half of the medium were replaced every 3-4 days. Finally, after 14 days of coculture cells were harvested and assessed by flow cytometry for B and myeloid lineage differentiation.

Supplemental Figures



Suppl. Fig 1a: *Validation of differential gene expression by Q-PCR. Sorted LSK cells were cultured and transduced with CRE-GFP as described in the supplemental experimental procedures. RNA was isolated and used for analysis by Q-PCR for the indicated Wnt target genes.*

Suppl. Fig 1b: *Biological processes associated with clusters 1 and 2. For details see text*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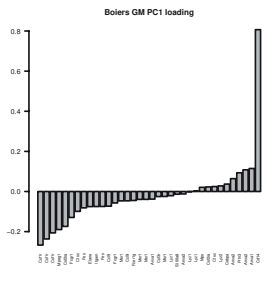
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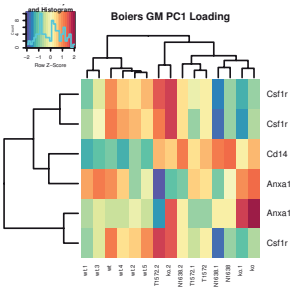
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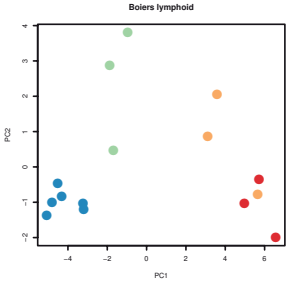
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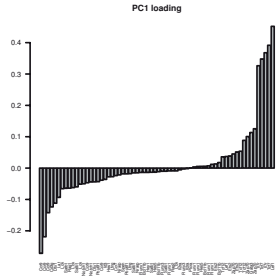
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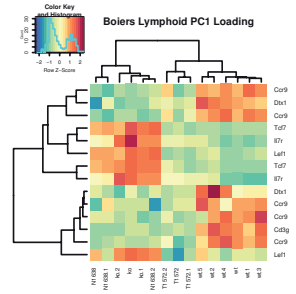
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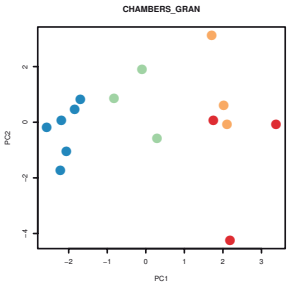
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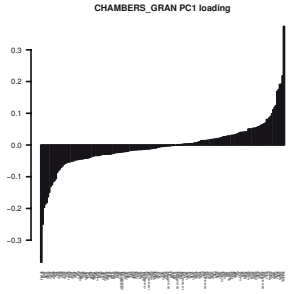
PC1
PC2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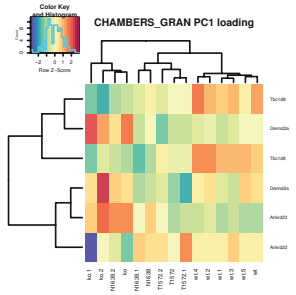
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Row Z-Score
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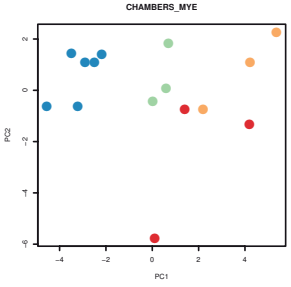
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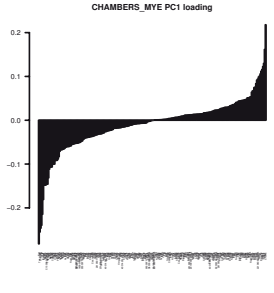
PC1
PC2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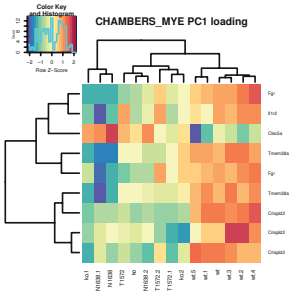
Color Key and Histogram
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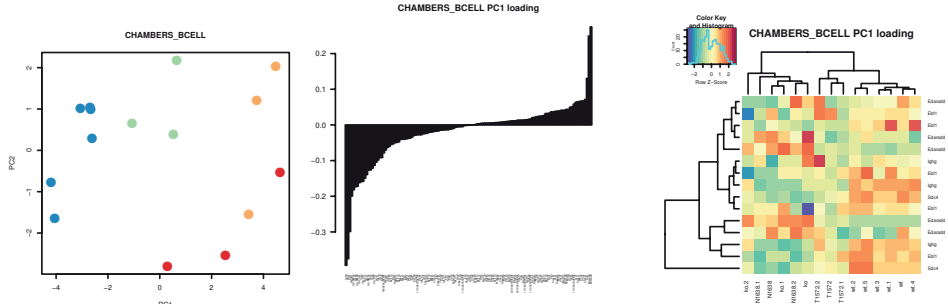
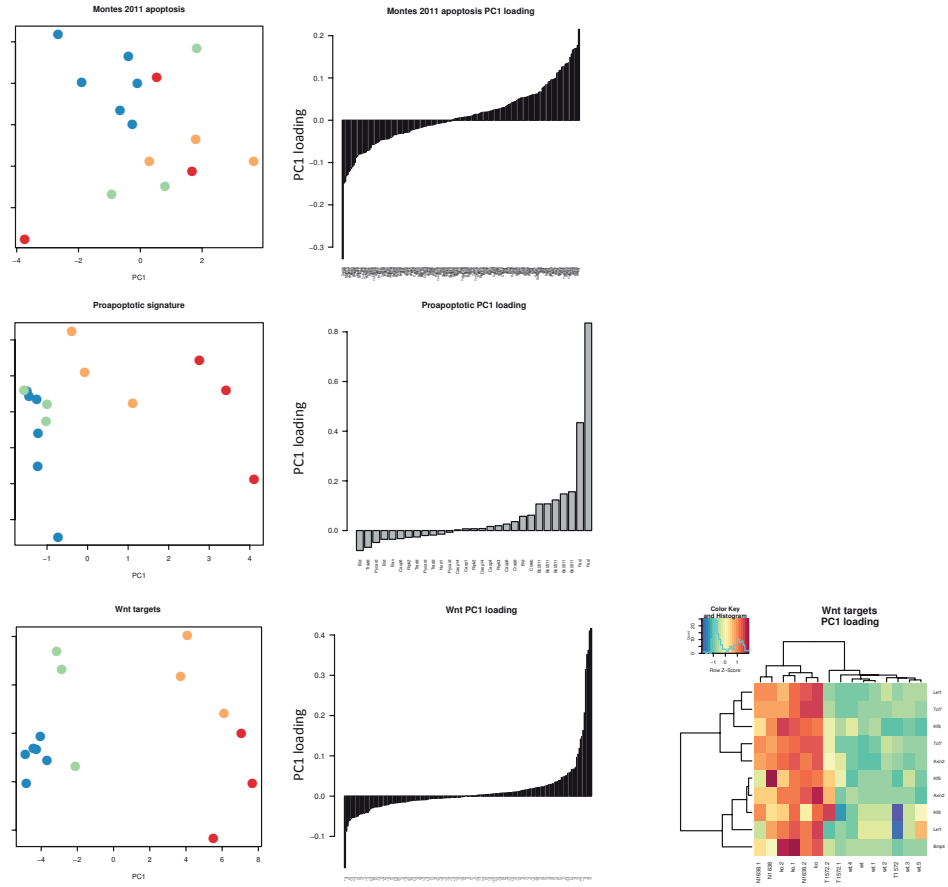


Suppl. Fig 2: *High Wnt signaling is associated with differentiation into monocytes and B lymphocytes based on published gene sets.*

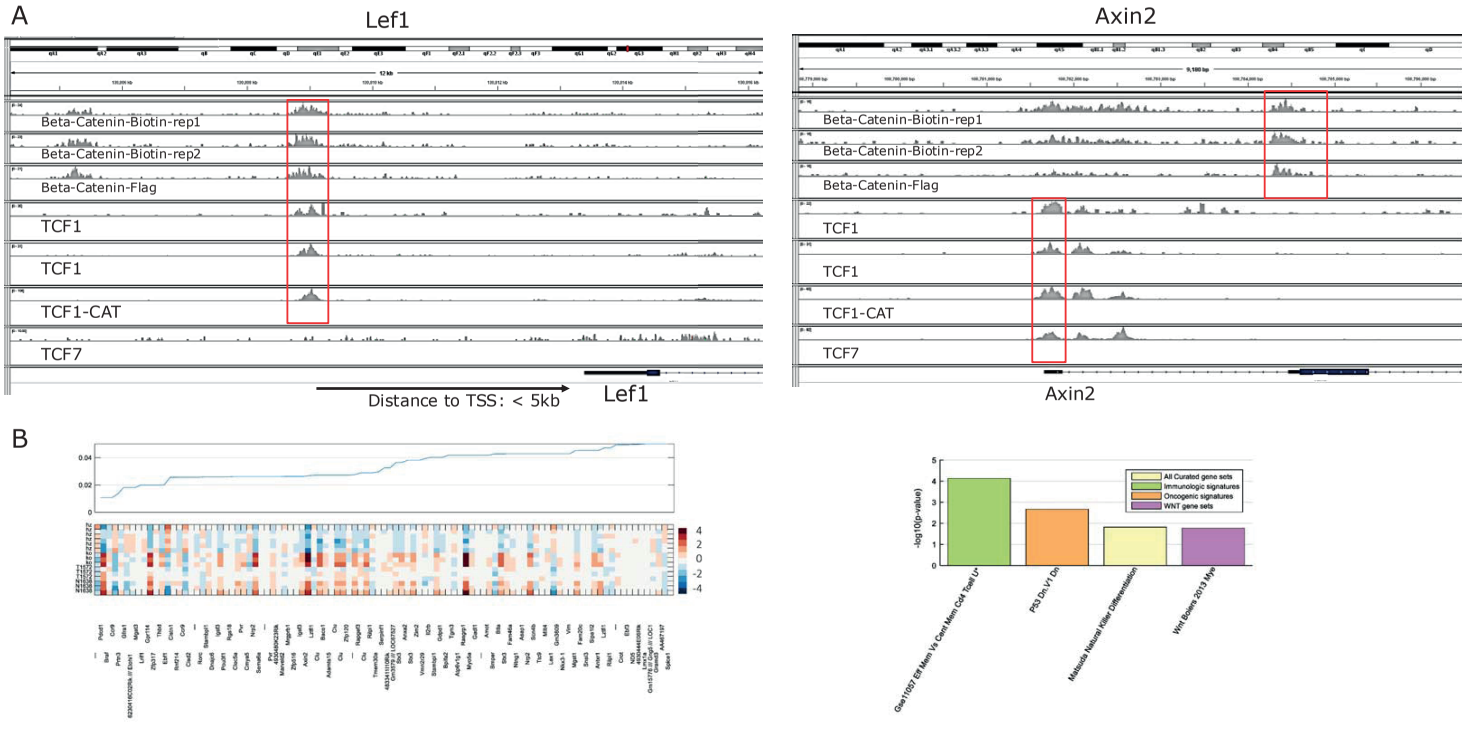


Suppl. Fig 3: *No differences in apoptosis and cell cycle in high Wnt signature when compared to published gene sets.*

Table S1

Gene-set	Pathway	P_{BY}<0.05	Genes
All Curated gene sets	MATSUDA_NATURAL_KILLER_DIFFERENTIATION	6.58E-06	ANXA1,APCDD1,CCR9,CD160,CDC23,GPR34,IL2RB,MYO5A,NTRK3,PDCD1,PLAGL1,PRSS23,PTPRF,PVR,SH3BGRL2,SYTL2,TCF7,TULP3,XCL1,ZC3H12C
All Curated gene sets	PICCALUGA_ANGIOIMMUNOBLASTIC_LYMPHOMA_UP	0.0002209	ANTXR1,CD93,CLU,DCLK1,FN1,FSTL1,IL18,LAMC1,PLA2G4C,RAI14,TMEM163,TNS1
All Curated gene sets	LIU_PROSTATE_CANCER_DN	0.0002209	BEND5,CHST2,CLU,CRISPLD2,EPAS1,GPR155,ITGA2,PHLDA1,PLAGL1,PRSS23,RHOJ,ROBO1,TFCP2L1,TMEM35,TNS1,WIF1,ZCCHC14
All Curated gene sets	ONDER_CDH1_TARGETS_2_DN	0.0002209	ALDH1A3,CD83,CDK5R1,EPAS1,FGD6,FST,GJB5,IGSF3,IL18,ITGA2,KLF5,PTPRF,ROBO1,TFCP2L1,THBD,TNFRSF25,TPD52L1
All Curated gene sets	DELYS_THYROID_CANCER_UP	0.0006965	ALDH1A3,ANXA1,CHST2,DPP4,ENTPD1,FN1,IGSF3,ITGA2,MED13,NRP2,NT5E,P4HA2,PRSS23,PTPRF,S100A5,STX3
All Curated gene sets	ST_WNT_BETA_CATENIN_PATHWAY	0.001009	APC,AXIN2,DKK2,FSTL1,NKD1,WIF1
All Curated gene sets	SANA_TNF_SIGNALING_DN	0.001009	ANTXR1,ANXA1,CLU,EPAS1,GIMAP6,NT5E,PHLDA1,RHOJ
All Curated gene sets	GOZGIT_ESR1_TARGETS_DN	0.001305	ABHD2,CLU,DCLK1,FETUB,GFRA1,GPC4,MB21D2,MYO5A,PPAP2A,PRSS23,RASGRP1,RNF144B,SDK1,SH3BGRL2,SHROOM3,SIPA1L2,SYTL2,THBD,THSD4
All Curated gene sets	CUI_TCF21_TARGETS_2_UP	0.001305	ANTXR1,APCDD1,ARSB,BMP4,BMPER,CLU,DCLK1,EMID1,FN1,GAS2L3,HUNK,KLF5,LYPD6B,NKD1,NRP2
All Curated gene sets	GAVIN_PDE3B_TARGETS	0.001305	ENTPD1,IL18,LAMC1,NT5E,SYTL2
All Curated gene sets	NABA_MATRISOME	0.003449	ADAM22,ANXA1,BMP4,BMPER,CRISPLD2,ELFN1,EMID1,FN1,FREM2,FST,FSTL1,GPC4,IL18,ISM1,KY,LAMC1,P4HA2,S100A5,SCUBE3,THSD4,WIF1,XCL1
All Curated gene sets	KEGG_WNT_SIGNALING_PATHWAY	0.00359	APC,AXIN2,CAMK2D,DKK2,LEF1,NFATC2,NKD1,TCF7,WIF1
All Curated gene sets	CERVERA_SDHB_TARGETS_1_UP	0.00359	CAND2,CCDC109B,FSTL1,IL18,LYPD6B,PACSIN1,PRSS23,TNFRSF19
All Curated gene sets	KINSEY_TARGETS_OF_EWSR1_FLI1_FUSION_DN	0.005222	CAMK2D,DCLK1,DLC1,FAM63A,FN1,FSTL1,LAMC1,MB21D2,NT5E,PHLDA1,PRSS23,SIPA1L2
All Curated gene sets	CHARAFE_BREAST_CANCER_LUMINAL_VS_BASAL_DN	0.005292	ADA,ALDH1A3,ANTXR1,ANXA1,CD14,FST,FSTL1,IL18,IL7R,KLF5,LAMC1,NT5E,PHLDA1,ZC3H12C
All Curated gene sets	RIGGI_EWING_SARCOMA_PROGENITOR_DN	0.007768	ABHD2,ALDH1A3,BACE1,BMP4,CLU,FST,NRP2,PHLDA1,TNFRSF19
All Curated gene sets	SANSOM_WNT_PATHWAY_REQUIRE_MYC	0.008652	AXIN2,LEF1,NKD1,TCF7,TNFRSF19,WIF1
All Curated gene sets	PASQUALUCCI_LYMPHOMA_BY_GC_STAGE_UP	0.008714	ADA,ANTXR1,ENTPD1,IRF4,NUDT4,OSBPL1A,PHLDA1,PVR,SH3BGRL2,SHROOM3,TULP3
All Curated gene sets	BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL_TRANS	0.008714	AXIN2,CD14,CHST2,EFHD1,EXTL3,FAM63A,GFRA1,GPC4,IL2RB,KIF5C,LEF1,MYO5A,PDCD1,STX3,SULT1A1,TEK,TFCP2L1,THBD,TULP3,XCL1
All Curated gene sets	GAUSSMANN_MLL_AF4_FUSION_TARGETS_F_UP	0.009692	ARHGAP28,ARSB,BMP4,BMPER,FST,GPC4,IL18,NT5E,TEK
All Curated gene sets	GAVIN_FOXP3_TARGETS_CLUSTER_P4	0.01055	CCDC109B,CD83,EPAS1,IL2RB,LYPD6B,PLAGL1,SH3BGRL2
All Curated gene sets	KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.01299	CCR2,CCR9,EDAR,IL17RB,IL18,IL2RB,IL7R,TNFRSF19,TNFRSF25,XCL1
All Curated gene sets	DODD_NASOPHARYNGEAL_CARCINOMA_UP	0.01525	ABHD2,ALDH1A3,ANXA1,APCDD1,ATP13A4,BACE1,BDH1,BEND5,CAND2,CLU,EDAR,EPAS1,FAM63A,FMN1,IL18,KLF5,LYPD6B,PRKAA2,PRSS23,SH3BGRL2,SMPD3,SNX31,SYTL2,TNFRSF19,TNS1,TUBB3
All Curated gene sets	CREIGHTON_ENDOCRINE_THERAPY_RESISTANCE_1	0.01646	BMPER,CCDC101,CRISPLD2,DLC1,EFHD1,FREM2,GFRA1,ITGA2,MB21D2,MYO5A,PRSS23,SYTL2,THSD4,TPD52L1
All Curated gene sets	SMID_BREAST_CANCER_NORMAL_LIKE_UP	0.01763	CCR2,CD3G,CLU,DPP4,GIMAP6,IL7R,LEF1,NT5E,SNCAIP,THBD,TNFRSF25,WIF1,XCL1
All Curated gene sets	SMIRNOV_CIRCULATING_ENDOTHELIOCYTES_IN_CANCER_UP	0.01851	B4GALT5,CD14,CD93,CLU,EPAS1,PRSS23,THBD,TNS1
All Curated gene sets	WALLACE_PROSTATE_CANCER_RACE_UP	0.01852	CCDC109B,CD83,CD93,CLU,DLC1,GIMAP6,IL7R,RASGRP1,THBD,TMEM35
All Curated gene sets	OI_PLASMACYTOMA_UP	0.02286	CARD11,CCR2,CD3G,CLU,DPP4,IL17RB,IL18,IL2RB,TUBB3,XCL1
All Curated gene sets	AMIT_EGF_RESPONSE_480_HELA	0.02337	ABHD2,DCLK1,FST,ITGA2,NUDT4,PTPRF,PVR,TUBB3
All Curated gene sets	NABA_MATRISOME_ASSOCIATED	0.03018	ADAM22,ANXA1,BMP4,ELFN1,FREM2,FST,FSTL1,GPC4,IL18,ISM1,KY,P4HA2,S100A5,SCUBE3,WIF1,XCL1
All Curated gene sets	REACTOME_IMMUNE_SYSTEM	0.03141	BTLA,CAMK2D,CARD11,CCR2,CD14,CD160,CD3G,CDC23,IL18,IL2RB,IL7R,IRF4,OSBPL1A,PDCD1,PVR,RAP1GAP2,RASGRP1,RNF144B
All Curated gene sets	FULCHER_INFLAMMATORY_RESPONSE_LECTIN_VS_LPS_UP	0.03141	ABHD2,CD93,CHST2,FN1,IL7R,IRF4,MB21D2,MYO5A,NRIP3,P4HA2,PHLDA1,RAI14,RASGRP1,THBD
All Curated gene sets	SCHAEFFER_PROSTATE_DEVELOPMENT_48HR_UP	0.03313	ALDH1A3,ANXA1,BDH1,CLU,CRISPLD2,EDARADD,GPR155,NT5E,PPFIBP2,SULT1A1,TFCP2L1,TPD52L1,WIF1
All Curated gene sets	KIM_MYC_AMPLIFICATION_TARGETS_DN	0.03336	DCLK1,GAS2L3,IL17RB,KLF5,NFATC2,SHROOM3
All Curated gene sets	LIM_MAMMARY_STEM_CELL_UP	0.04392	ANTXR1,EDARADD,EPAS1,FST,ISM1,LAMC1,NRP2,NT5E,PPAP2A,RHOJ,THSD1,TNS1,WIF1
All Curated gene sets	KEGG_BASAL_CELL_CARCINOMA	0.04862	APC,AXIN2,BMP4,LEF1,TCF7
All Curated gene sets	TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_DN	0.04862	ANTXR1,CD14,CLU,ENTPD1,EPAS1,GPR34,IL7R,TMEM163
All Curated gene sets	LINDGREN_BLADDER_CANCER_CLUSTER_2B	0.0488	CRISPLD2,EFHD1,ENTPD1,IL7R,LEF1,MYO5A,NRP2,TBC1D8,TCF7,THBD,TNS1
All Curated gene sets	NUYTTEN_EZH2_TARGETS_UP	0.0488	ANXA1,AXIN2,B4GALT5,BACE1,CCDC109B,CD83,FGD6,FN1,GPR155,NT5E,P4HA2,PLAGL1,PRRG1,PTPRF,ROBO1,STX3,TCF7,THSD1,ZC3H12C
All Curated gene sets	SCHAEFFER_PROSTATE_DEVELOPMENT_48HR_DN	0.0488	ANTXR1,CD83,CHDH,DKK2,GAS2L3,HUNK,LYPD6B,NRP2,P4HA2,PRTG,RHOJ,SIPA1L2
Computational gene sets	MODULE_46	2.83E-06	ADA,CCR2,CCR9,CD14,CD3G,CD83,CDK5R1,CLU,DPP4,ENTPD1,FN1,IL18,IL2RB,IL7R,P4HA2,PDCD1,XCL1
Computational gene sets	MODULE_75	2.83E-06	ADA,CCR2,CCR9,CD14,CD3G,CD83,CDK5R1,CLU,DPP4,FN1,IL18,IL2RB,IL7R,P4HA2,PDCD1,TEK,XCL1
Gene ontology (GO)	PLASMA_MEMBRANE_PART	0.000438	ACTN2,APC,BACE1,CACNA1B,CACNA1D,CARD11,CCR2,CCR9,CD160,CD83,DCLK1,ENTPD1,GPC4,GPR34,IL17RB,IL2RB,ITGA2,NTRK3,PRRG1,PTPRF,ROBO1,SHROOM3,STX3,SYTL2,TEK,THBD,TNFRSF25
Gene ontology (GO)	MEMBRANE	0.0006684	ACTN2,APC,BACE1,CACNA1B,CACNA1D,CARD11,CCR2,CCR9,CD14,CD160,CD83,CDK5R1,DCLK1,ENTPD1,GPC4,GPR114,GPR34,IL17RB,IL2RB,ITGA2,NTRK3,PLA2G4C,PPAP2A,PRRG1,PTPRF,PVR,RNF144B,ROBO1,SHROOM3,SLC7A4,STX3,SYTL2,TBC1D8,TEK,THBD,TNFRSF25
Gene ontology (GO)	PLASMA_MEMBRANE	0.0007698	ACTN2,APC,BACE1,CACNA1B,CACNA1D,CARD11,CCR2,CCR9,CD14,CD160,CD83,DCLK1,ENTPD1,GPC4,GPR34,IL17RB,IL2RB,ITGA2,NTRK3,PPAP2A,PRRG1,PTPRF,ROBO1,SHROOM3,STX3,SYTL2,TEK,THBD,TNFRSF25
Gene ontology (GO)	MEMBRANE_PART	0.001722	ACTN2,APC,BACE1,CACNA1B,CACNA1D,CARD11,CCR2,CCR9,CD160,CD83,DCLK1,ENTPD1,GPC4,GPR114,GPR34,IL17RB,IL2RB,ITGA2,NTRK3,PRRG1,PTPRF,PVR,RNF144B,ROBO1,SHROOM3,SLC7A4,STX3,SYTL2,TEK,THBD,TNFRSF25
Gene ontology (GO)	SIGNAL_TRANSDUCTION	0.007247	ANXA1,AXIN2,CCR2,CCR9,CD14,CD160,CD3G,CD83,CDK5R1,DLC1,EDARADD,EPAS1,FGD6,GPR34,HUNK,IL2RB,IL7R,KCNIP2,MED13,NTRK3,NUDT4,PPAP2A,PRKAA2,PTPRF,RASGRP1,TEK,TNFRSF25,TPD52L1,XCL1
Gene ontology (GO)	RESPONSE_TO_EXTERNAL_STIMULUS	0.01613	AHSG,ANXA1,CCR2,CCR9,CHST2,ENTPD1,ITGA2,PGLYRP2,SHROOM3,THBD,XCL1
Gene ontology (GO)	RECEPTOR_ACTIVITY	0.01613	CD14,CD160,CD3G,GFRA1,GPR114,IL2RB,IL7R,MED13,NRP2,PGLYRP2,PTPRF,PVR,ROBO1,TEK,TNFRSF25
Hallmark gene sets	HALLMARK_ESTROGEN_RESPONSE_EARLY	0.004103	ABHD2,DLC1,FAM63A,GFRA1,IL17RB,PRSS23,RASGRP1,THSD4,TPD52L1
Hallmark gene sets	HALLMARK_INFLAMMATORY_RESPONSE	0.006397	CD14,CHST2,IL18,IL2RB,IL7R,PVR,RASGRP1,RNF144B
Hallmark gene sets	HALLMARK_COAGULATION	0.006397	ANXA1,CLU,DPP4,FN1,ITGA2,PRSS23,THBD
Hallmark gene sets	HALLMARK_IL2_STAT5_SIGNALING	0.006397	CD83,IL2RB,IRF4,NT5E,PHLDA1,PLAGL1,PPAP2A,SH3BGRL2
Hallmark gene sets	HALLMARK_WNT_BETA_CATENIN_SIGNALING	0.01107	AXIN2,LEF1,NKD1,TCF7
Hallmark gene sets	HALLMARK_COMPLEMENT	0.01636	ACTN2,CDK5R1,CLU,DPP4,FN1,KCNIP2,RASGRP1
Immunologic signatures	GSE20366_EX_VIVO_VS_DEC205_CONVERSION_NAIVE_CD4_TCELL_UP	0.001054	ACTN2,CCR2,CD160,EPAS1,GPR114,GPR34,IL17RB,RASGRP1,THBD,XCL1,XKRX
Immunologic signatures	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN2_THYMOCYTE_DN	0.001054	ADA,AXIN2,CCDC109B,CD3G,DPP4,EDARADD,IL17RB,IL7R,PDCD1,PTPRF,TUBB3
Immunologic signatures	GSE7852_TREG_VS_TCONV_LN_UP	0.001054	CCR2,CD83,ENTPD1,FGD6,IRF4,LAMC1,NT5E,PLAGL1,PPAP2A,ZC3H12C,ZDHHC23
Immunologic signatures	GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_BCELL_UP	0.003344	ANXA1,CCDC109B,CD3G,DPP4,GIMAP6,IL7R,LEF1,TCF7,TNFRSF25,ZCCHC14
Immunologic signatures	GSE30962_PRIMARY_VS_SECONDARY_CHRONIC_LCMV_INF_CD8_TCELL	0.003344	AHSG,ANXA1,CD93,ENTPD1,EPAS1,GPR114,GPR34,PRKAA2,RASGRP1,TMEM163
Immunologic signatures	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN2_THYMOCYTE_ADULT_	0.003403	AXIN2,CCDC109B,CD3G,EDARADD,IL17RB,IL7R,LEF1,PPAP2A,PTPRF,SYTL2
Immunologic signatures	GSE7460_TCONV_VS_TREG_LN_DN	0.004089	CD83,DPP4,ENTPD1,IL2RB,IRF4,NT5E,PPAP2A,SH3BGRL2,ZC3H12C,ZDHHC23
Immunologic signatures	GSE20366_EX_VIVO_VS_DEC205_CONVERSION_NAIVE_CD4_TCELL_DN	0.0137	ANXA1,CCDC109B,CD83,DCLK1,IL18,PLAGL1,RNF144B,SNX31,STX3
Immunologic signatures	GSE10325_CD4_TCELL_VS_BCELL_UP	0.01559	ANXA1,CCR2,CD3G,DPP4,GIMAP6,IL2RB,LEF1,RASGRP1,TNFRSF25
Immunologic signatures	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN3_THYMOCYTE_DN	0.01559	ADA,CCDC109B,GFRA1,IL7R,LEF1,PDCD1,PTPRF,TUBB3,TULP3
Immunologic signatures	GSE39820_CTRL_VS_IL1B_IL6_IL23A_CD4_TCELL_UP	0.01559	CD83,GAS2L3,GPR34,LYPD6B,PACSIN1,PHLDA1,PLAGL1,RASGRP1,SYTL2
Immunologic signatures	GSE7460_TCONV_VS_TREG_THYMUS_DN	0.01559	CCDC109B,CD83,IGSF3,IL2RB,KIF5C,NRP2,PLAGL1,PPAP2A,SH3BGRL2
Immunologic signatures	GSE7852_TREG_VS_TCONV_THYMUS_UP	0.01634	CCDC109B,CCR2,CD83,IGSF3,KIF5C,PDCD1,PLAGL1,PPAP2A,SH3BGRL2
Immunologic signatures	GSE24142_DN2_VS_DN3_THYMOCYTE_DN	0.01649	CCR9,D3G,GFRA1,IRF4,LEF1,PHLDA1,PTPRF,TBC1D8,TULP3
Immunologic signatures	GSE3982_BCELL_VS_CENT_MEMORY_CD4_TCELL_DN	0.03801	DLC1,DPP4,IL7R,ITGA2,PHLDA1,PRKAA2,TCF7,TNS1
Immunologic signatures	GSE3982_MEMORY_CD4_TCELL_VS_BCELL_UP	0.04586	ANXA1,CAND2,CD3G,DPP4,GIMAP6,IL2RB,KIF5C,PHLDA1
Motif gene sets	TTGTTT_V$FOXO4_01	0.0001493	ANXA1,APC,AXIN2,BDH1,BMP4,CCDC109B,CD83,DLC1,EDAR,ENTPD1,EXTL3,FAM63A,FN1,FSTF,STL1,GFRA1,IL18,IL7R,IRF4,ITGA2,KCNIP2,KLF5,LAMC1,NKD1,NRP2,NTRK3,RNF214,ROBO1,SCUBE3,SDK1,SMPD3,SNCAIP,SYTL2,TEK,TNFRSF19,XKRX,ZCCHC14
Motif gene sets	CTTTGA_V$LEF1_Q2	0.0001493	ABHD2,ATP13A4,BACE1,CD160,CPB1,DLC1,EMID1,FAM63A,FST,GFRA1,GPC4,KIF7,KY,LEF1,MB21D2,MED13,NKD1,NRP2,ROBO1,SESTD1,SLC22A23,SNCAIP,SYTL2,TBC1D8,TCF7,TNFRSF19,XKRX
Motif gene sets	TGGAAA_V$NFAT_Q4_01	0.006435	ANTXR1,B4GALT5,BMP4,CHDH,DCLK1,DKK2,DLC1,EFHD1,FN1,FST,FSTL1,GALNT7,IGSF3,IL17RB,IL7R,IRF4,ITGA2,KCNIP2,KLF5,MED13,NFATC2,SCUBE3,SH3BGRL2,SMPD3,SNCAIP,TBC1D8,TMEM163,TMEM35,TNFRSF19,TNS1,XKRX
Motif gene sets	V$TCF4_Q5	0.009151	ABHD2,FAM63A,FST,GPC4,KY,NKD1,NRP2,SYTL2,TCF7,TNFRSF19
Oncogenic signatures	CAMP_UP.V1_DN	1.97E-05	ANXA1,BACE1,CAMK2D,CCR9,CD160,CD83,CHST2,FSTL1,IL7R,NFATC2,TUBB3,ZCCHC14
Oncogenic signatures	MEL18_DN.V1_UP	0.03587	CD83,CHST2,CRISPLD2,IL7R,NRP2,NT5E,TEK

Table S2				
Experiment	**Study**	**Significantly detected binding regions**	**Mapped to genes within 5Kb from TSS**	**P-value, Significance with WNT-associated genesets from MsigDB**
TCF1	GSE52070	591	116	0.033
TCF1 (n=2)	GSE46662	732 in Sample 1	131	0.050
		2600 in Sample 2	653	9.395E-04
TCF7	GSE31221	6395	2015	0.017
Beta-Catenin (n=3)	GSE43565	990 in Sample 1	121	2.273E-04
		385 in Sample 2	49	9.564E-04
		671 in Sample 3	79	0.004



Suppl. Fig 4a: *Tcf and beta catenin binding sites in the Lef1 and Axin2 promoters based on literature data mining of CHIP-Seq data*

Suppl. Fig 4b: *Differentially expressed genes from the gene expression profiles of heterozygote samples against 1572T,1638N, and full KO, and detected in A. 157 differential expressed genes with P<0.05 and B. the representation of 55 unique gene sets (see also Table S3)*

Table S3

Gene-set	Pathway	P_{BY}≤0.05	Genes
WNT gene sets	WNT_BOIERS_2013_LYMPHOID	0.01053	CCR9,LEF1,TCF7
All Curated gene sets	ST_WNT_BETA_CATENIN_PATHWAY	0.0001483	APC,AXIN2,DKK2,FSTL1,NKD1,WIF1
All Curated gene sets	RIGGI_EWING_SARCOMA_PROGENITOR_DN	0.0006029	ALDH1A3,BACE1,BMP4,CLU,EBF1,FST,NRP2,PHLDA1,TNFRSF19
All Curated gene sets	SANSOM_WNT_PATHWAY_REQUIRE_MYC	0.001274	AXIN2,LEF1,NKD1,TCF7,TNFRSF19,WIF1
All Curated gene sets	LIU_PROSTATE_CANCER_DN	0.001855	BEND5,CHST2,CLU,EPAS1,ITGA2,NDNF,PHLDA1,PRSS23,ROBO1,TFCP2L1,WIF1,ZCCHC14
All Curated gene sets	MATSUDA_NATURAL_KILLER_DIFFERENTIATION	0.001855	ANXA1,APCDD1,CCR9,CD160,EBF1,NTRK3,PDCD1,PRSS23,SH3BGRL2,TCF7,TULP3,ZC3H12C
All Curated gene sets	KINSEY_TARGETS_OF_EWSR1_FLII_FUSION_DN	0.001979	DCLK1,EBF1,FAM63A,FSTL1,IL17RD,MB21D2,NT5E,PHLDA1,PRSS23,SIPA1L2
All Curated gene sets	KUMAR_TARGETS_OF_MLL_AF9_FUSION	0.001979	ANXA1,CCR9,CD83,EBF1,EXTL3,GPC4,IL7R,IRF4,LEF1,TCF7,TNFRSF19
All Curated gene sets	CUI_TCF21_TARGETS_2_UP	0.003067	ANTXR1,APCDD1,ARSB,BMP4,CLU,DCLK1,HUNK,KLF5,LYPD6B,NKD1,NRP2
All Curated gene sets	CHARAFE_BREAST_CANCER_LUMINAL_VS_BASAL_DN	0.003672	ADA,ALDH1A3,ANTXR1,ANXA1,FST,FSTL1,IL7R,KLF5,NT5E,PHLDA1,ZC3H12C
All Curated gene sets	DODD_NASOPHARYNGEAL_CARCINOMA_UP	0.003672	ALDH1A3,ANXA1,APCDD1,ATP13A4,BACE1,BDH1,BEND5,CLU,EBF1,EDAR,EPAS1,FAM63A,KLF5,LYPD6B,PRKAA2,PRSS23,SH3BGRL2,TNFRSF19,TUBB3,UST,WWC1
All Curated gene sets	DELYS_THYROID_CANCER_UP	0.003672	ALDH1A3,ANXA1,CHST2,DPP4,IGSF3,ITGA2,MED13,NRP2,NT5E,PRSS23,STX3
All Curated gene sets	ONDER_CDH1_TARGETS_2_DN	0.004116	ALDH1A3,CD83,EPAS1,FST,IGSF3,ITGA2,KLF5,ROBO1,TFCP2L1,THBD,WWC1
All Curated gene sets	SANA_TNF_SIGNALING_DN	0.004116	ANTXR1,ANXA1,CLU,EPAS1,NT5E,PHLDA1
All Curated gene sets	KEGG_WNT_SIGNALING_PATHWAY	0.004871	APC,AXIN2,DKK2,LEF1,NKD1,TCF7,WIF1
All Curated gene sets	KEGG_BASAL_CELL_CARCINOMA	0.005932	APC,AXIN2,BMP4,LEF1,TCF7
All Curated gene sets	CUI_TCF21_TARGETS_2_DN	0.006368	ANXA1,ARHGAP28,BACE1,DKK2,DPP4,EBF1,EPAS1,MED13,NT5E,PPAP2A,SH3BGRL2,SHROOM3,SNCAIP,THBD
All Curated gene sets	BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL_TRANS	0.01287	AXIN2,CHST2,EFHD1,EXTL3,FAM63A,GPC4,KIF5C,LEF1,PDCD1,STX3,SULT1A1,TFCP2L1,THBD,TULP3
All Curated gene sets	GAUSSMANN_MLL_AF4_FUSION_TARGETS_F_UP	0.0141	ARHGAP28,ARSB,BMP4,FST,GPC4,IL17RD,NT5E
All Curated gene sets	CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_DN	0.01879	ANTXR1,ANXA1,CHST2,FST,FSTL1,IL7R,NT5E,PHLDA1,RAI14,ZC3H12C
All Curated gene sets	GOZGIT_ESR1_TARGETS_DN	0.03962	CLU,DCLK1,FETUB,GPC4,MB21D2,PPAP2A,PRSS23,RASGRP1,SH3BGRL2,SHROOM3,SIPA1L2,THBD
All Curated gene sets	ENK_UV_RESPONSE_EPIDERMIS_DN	0.04184	ANXA1,APC,CD83,ITGA2,PHLDA1,PPAP2A,PRSS23,RAI14,ROBO1,THBD
All Curated gene sets	WNT_SIGNALING	0.04194	APC,LEF1,NKD1,TCF7,WIF1
All Curated gene sets	PID_PS1_PATHWAY	0.04605	APC,DKK2,NKD1,WIF1
All Curated gene sets	SENESE_HDAC1_AND_HDAC2_TARGETS_UP	0.04605	DCLK1,DKK2,EXTL3,IL7R,NRIP3,PHLDA1,WWC1
All Curated gene sets	KIM_MYC_AMPLIFICATION_TARGETS_DN	0.04605	DCLK1,IL17RB,IL17RD,KLF5,SHROOM3
Motif gene sets	TTGTTT_V$FOXO4_01	3.15E-05	ANXA1,APC,AXIN2,BDH1,BMP4,CCDC109B,CD83,EBF1,EDAR,EXTL3,FAM63A,FST,FSTL1,IL7R,IRF4,ITGA2,KCNIP2,KLF5,NKD1,NRP2,NTRK3,RNF214,ROBO1,SNCAIP,TNFRSF19,XKRX,ZCCHC14
Motif gene sets	CTTTGA_V$LEF1_Q2	0.0003071	ATP13A4,BACE1,CD160,CPB1,FAM63A,FST,GPC4,KY,LEF1,MB21D2,MED13,NKD1,NRP2,ROBO1,SLC22A23,SNCAIP,TCF7,TNFRSF19,XKRX
Motif gene sets	CAGGTG_V$E12_Q6	0.001973	ACTN2,AXIN2,BACE1,BMP4,CD83,CPB1,EBF1,EDAR,EPAS1,EXTL3,FST,IGSF3,ITGA2,KCNIP2,LEF1,LYPD6B,MB21D2,NRIP3,NRP2,NTRK3,SH3BGRL2,SNCAIP,TCF7,UNC45B,UST,WWC1
Motif gene sets	V$TCF4_Q5	0.005048	FAM63A,FST,GPC4,KY,NKD1,NRP2,TCF7,TNFRSF19
Motif gene sets	TGGAAA_V$NFAT_Q4_01	0.006748	ANTXR1,BMP4,DCLK1,DKK2,EBF1,EFHD1,FST,FSTL1,IGSF3,IL17RB,IL7R,IRF4,ITGA2,KCNIP2,KLF5,MED13,SH3BGRL2,SNCAIP,TMEM163,TNFRSF19,XKRX
Motif gene sets	TATTATA,MIR-374	0.01408	ARHGAP28,BACE1,CHST2,EDAR,MED13,RNF214,UST,ZCCHC14
Motif gene sets	TGCCAAR_V$NF1_Q6	0.01468	AHSG,AXIN2,DCLK1,KY,LEF1,MB21D2,MED13,NRP2,NTRK3,RAI14,ROBO1,XKRX
Motif gene sets	RTAAACA_V$FREAC2_01	0.02911	AXIN2,BMP4,FST,FSTL1,IRF4,KY,NTRK3,ROBO1,SNCAIP,TCF7,TNFRSF19,UNC45B,UST
Oncogenic signatures	CAMP_UP.V1_DN	5.78E-06	ANXA1,BACE1,CCR9,CD160,CD83,CHST2,FSTL1,IL7R,TUBB3,ZCCHC14
Oncogenic signatures	AKT_UP.V1_DN	0.04519	AXIN2,EDARADD,TNFRSF19,TULP3,WIF1,ZC3H12C
Immunologic signature	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN2_THYMOCYTE_DN	0.001121	ADA,AXIN2,CCDC109B,DPP4,EDARADD,IL17RB,IL7R,PDCD1,TUBB3
Immunologic signature	GSE20366_EX_VIVO_VS_DEC205_CONVERSION_NAIVE_CD4_TCELL_UP	0.007534	ACTN2,CD160,EPAS1,GPR114,IL17RB,RASGRP1,THBD,XKRX
Immunologic signature	GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_BCELL_UP	0.01556	ANXA1,CCDC109B,DPP4,IL7R,LEF1,TCF7,ZCCHC14
Immunologic signature	GSE14350_IL2RB_KO_VS_WT_TREG_DN	0.01556	CCDC109B,CD160,CD83,KY,NT5E,PDCD1,ZC3H12C
Immunologic signature	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN3_THYMOCYTE_DN	0.01556	ADA,CCDC109B,IL7R,LEF1,PDCD1,TUBB3,TULP3
Immunologic signature	GSE24142_EARLY_THYMIC_PROGENITOR_VS_DN2_THYMOCYTE_ADULT_DN	0.01556	AXIN2,CCDC109B,EDARADD,IL17RB,IL7R,LEF1,PPAP2A
Immunologic signature	GSE26495_NAIVE_VS_PD1HIGH_CD8_TCELL_UP	0.01556	BDH1,BEND5,EDAR,EFHD1,LEF1,NT5E,PPAP2A
Immunologic signature	GSE26495_NAIVE_VS_PD1LOW_CD8_TCELL_UP	0.01556	BDH1,BEND5,EDAR,EFHD1,LEF1,NT5E,PPAP2A
Immunologic signature	GSE30962_PRIMARY_VS_SECONDARY_CHRONIC_LCMV_INF_CD8_TCELL_DN	0.01556	AHSG,ANXA1,EPAS1,GPR114,PRKAA2,RASGRP1,TMEM163
Immunologic signature	GSE3982_BCELL_VS_CENT_MEMORY_CD4_TCELL_DN	0.01556	DPP4,IL7R,ITGA2,NDNF,PHLDA1,PRKAA2,TCF7
Immunologic signature	GSE7460_TCONV_VS_TREG_LN_DN	0.01556	CD83,DPP4,IRF4,NT5E,PPAP2A,SH3BGRL2,ZC3H12C
Immunologic signature	GSE7460_TCONV_VS_TREG_THYMUS_DN	0.01556	CCDC109B,CD83,IGSF3,KIF5C,NRP2,PPAP2A,SH3BGRL2
Immunologic signature	GSE7852_TREG_VS_TCONV_THYMUS_UP	0.01556	CCDC109B,CD83,IGSF3,KIF5C,PDCD1,PPAP2A,SH3BGRL2
Hallmark gene sets	HALLMARK_WNT_BETA_CATENIN_SIGNALING	0.002609	AXIN2,LEF1,NKD1,TCF7
Hallmark gene sets	HALLMARK_COAGULATION	0.002609	ANXA1,CLU,DPP4,ITGA2,PRSS23,THBD
Hallmark gene sets	HALLMARK_IL2_STAT5_SIGNALING	0.007102	CD83,IRF4,NT5E,PHLDA1,PPAP2A,SH3BGRL2
Hallmark gene sets	HALLMARK_KRAS_SIGNALING_DN	0.007102	CHST2,CPB1,EDAR,EFHD1,PDCD1,TFCP2L1
Hallmark gene sets	HALLMARK_ESTROGEN_RESPONSE_EARLY	0.04111	FAM63A,IL17RB,PRSS23,RASGRP1,WWC1
Hallmark gene sets	HALLMARK_COMPLEMENT	0.04111	ACTN2,CLU,DPP4,KCNIP2,RASGRP1

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CHAPTER 3

The non-canonical Wnt receptor Ryk regulates hematopoietic stem cell repopulation in part by controlling proliferation and apoptosis

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Abstract

The development of blood and immune cells requires strict control by various signaling pathways in order to regulate self-renewal, differentiation and apoptosis in stem and progenitor cells. Recent evidence indicates critical roles for the canonical and non-canonical Wnt pathways in hematopoiesis. The non-canonical Wnt pathway is important for establishment of cell polarity and cell migration and regulates apoptosis in the thymus. We here investigate the role of the non-canonical Wnt receptor Ryk in hematopoiesis and lymphoid development. We show that there are dynamic changes in Ryk expression during development and in different hematopoietic tissues. Functionally, Ryk regulates NK cell development in a temporal fashion. Moreover, Ryk-deficient mice show diminished, but not absent self-renewal of hematopoietic stem cells (HSC), via effects on mildly increased proliferation and apoptosis. Thus, Ryk deficiency in HSCs from fetal liver reduces their quiescence, leading to proliferation-induced apoptosis and decreased self-renewal.

Introduction

In the bone marrow, blood cells develop from a small pool of hematopoietic stem cells (HSC).[1] This rare population of cells is located in a specific microenvironment, the niche, and endows HSCs with the capacity to self-renew and provides signals to further differentiate HSCs into all blood cell lineages.[2] A wide variety of signaling pathways regulate the fate of HSCs; in addition to these cells undergoing self-renewal or differentiation, they can also remain quiescent or undergo programmed cell death. These signaling pathways include Wnt, Notch, Hedgehog, BMP/SMAD, and many hematopoietic cytokines (SCF, TPO, angiopoietins).[3, 4] Defects in these pathways are implicated in the development of bone marrow failure syndromes and hematologic malignancies.[5]

Various subpopulations that are the progeny of stem cells can migrate from BM to thymus, where they develop into the T-cell lineage.[6] During thymic development, immature thymocytes gradually lose their proliferative and multi-lineage potential, and initiate a T-cell developmental program, a process called T-cell commitment.[7] Early stages of T-cell development are phenotypically characterized by the absence of mature T-cell markers CD4 and CD8. These stages are therefore collectively referred to as Double Negative (DN). In mice, DN stages are subdivided into four subpopulations termed DN1: CD44+ CD25−, DN2: CD44+ CD25+, DN3: CD44− CD25+, and DN4: CD44− CD25−. Afterwards, thymocytes develop to immature single positive stage defined as CD3− CD8+ to initiate T-cell receptor (TCR) rearrangement. Thymocytes with functional TCRs develop into the next stage, double positive for CD4 and CD8, and subsequently differentiate into either mature single positive (SP) CD4 or CD8 T cells,[8] which have different functional properties. CD4T cells provide help to other cells and CD8 T cells are cytotoxic.

In order to better understand processes that underlie the development of HSC into T cells, we and others have performed gene expression profiling of sorted subsets of HSCs, progenitor cell, and stages of T-cell differentiation.[9, 10, 11, 12] We focused on the Wnt signaling pathway, as it is required for both self-renewal of HSCs as well as for proper T-cell development in the thymus.

Wnt signaling pathways have historically been characterized as either canonical (Wnt/*β*-catenin pathway) or non-canonical pathways.[13, 14, 15, 16, 17] In the absence of canonical Wnt ligands and receptors, cytoplasmic levels of β-catenin are kept very low through the action of a protein complex (the so-called destruction complex) that actively targets β-catenin for degradation. Activation of the pathway by Wnt leads to inactivation of the destruction complex allowing buildup of the dephosphorylated form of *β*-catenin and its migration to the nucleus. In the nucleus, β-catenin binds to members of the TCF/LEF transcription

factor family, thereby converting them from transcriptional repressors into transcriptional activators. In the non-canonical pathways, that use Ca^{2+} signals or JNK kinases, there are no increases in *β*-catenin levels but cell polarity or motility, as well as regulation of apoptosis are the main biological effects.

We previously observed that Wnt ligands and receptors are very dynamically expressed in HSCs, progenitors, and thymocytes.[18] One of these genes encodes the non-canonical Wnt receptor Ryk. The Ryk (related to receptor tyrosine kinase), Ror (RTK-like orphan receptor), and MuSK (muscle-specific kinase) families of RTKs, which all have unexpected links to Wnt signaling, probably use a unique activation mechanism.[19] Ryk contains a Wnt-inhibitory factor-1 domain in its extracellular region and was hypothesized to function as a receptor (or co-receptor) for Wnts, but lacks endogenous PTK activity owing to mutations in the kinase domain.[20] The first function of Ryk was uncovered in a screen for genes involved in Drosophila CNS axon pathfinding[21] and as a gene required for learning and memory in flies. A further breakthrough in understanding Ryk function came from the finding that Ryk is a axon-repulsive receptor for the WNT5 protein.[22] Most of the fly and mammalian Ryk studies to date have focused on its role in aspects of the developing or regenerating nervous system (reviewed in ref. 23), although the Ryks also have roles in other tissues. Ryk directly binds Wnt-1 and Wnt3a via its WIF domain and forms a ternary complex with Frizzled required for the induction neurite outgrowth in dorsal root ganglia explants.[24] The intracellular domain of Ryk binds to disheveled, which is required for TCF activation in response to Wnt3a activation.

There is only a handful of studies focused on the role of Ryk in hematopoiesis and thymopoiesis. More than 20 years ago, expression analyses revealed that Ryk is regulated during hematopoietic development and stages of maturation.[25] More recently, it was proposed that Wnt5a regulates HSC quiescence and hematopoietic repopulation through the Ryk receptor and that this process is mediated by suppression of reactive oxygen species.[26] We recently showed that canonical and non-canonical Wnt signaling have vastly different and contrasting roles in hematopoiesis and thymopoiesis, in part, by regulating cell survival and apoptosis.[27]

None of the few previous studies on Ryk function in hematopoiesis employed genetic loss-of-function models, hence we explored the role of RYK1 using mice, which have a targeted mutation in Ryk1 generated by knocking a lacZ allele into the coding region.[28] We analyzed the role of Ryk in four different experimental setups; (a) *ex vivo* functional gene expression analyses in neonatal mice and embryos, (b) *in vitro* assays for T-cell development in presence of the prototypical canonical and non-canonical Wnt ligands, Wnt3a, and Wnt5a, respectively,[27, 29] (c) primary *in vivo* murine bone marrow transplantation assays

(for blood cell reconstitution), and (d) secondary transplantation reconstitution assays to address self-renewal. Only subtle differences between the Ryk mutant and controls were observed in the first three assays. However, the secondary transplantation assay revealed that lack of Ryk results in lower stem cell repopulation indicating a role for Ryk in stem cell self-renewal. Our studies indicate that this is likely due to the fact that Ryk knock-out (KO) stem cells have diminished quiescence, leading to proliferation-induced apoptosis and decreased self-renewal.

Results

In order to assess gene expression patterns of Ryk in the murine hematopoietic systems, in particular during T-cell development, quantitative PCR was performed. First, we quantified Ryk expression in embryonic thymic lobes and fetal livers (FLs). Brain tissues were used as a positive control, as brain provides a rich source of Wnts and their receptors. The expression of Ryk was ~12-fold higher in FL, the site of hematopoiesis in the embryo, relative to the thymic lobes (Figure 1a). We also quantified Ryk expression during T-cell developmental stages in the adult murine thymus. The overall level of Ryk expression was much lower in the adult thymus compared with the embryonic thymic lobes. Nevertheless, the highest level of Ryk expression was observed at the most immature stage of DNs, and declined as thymocytes developed further. Notably, SP CD4+ T cells had a relatively higher Ryk expression compared with the SP CD8+ T cells (Figure 1b).

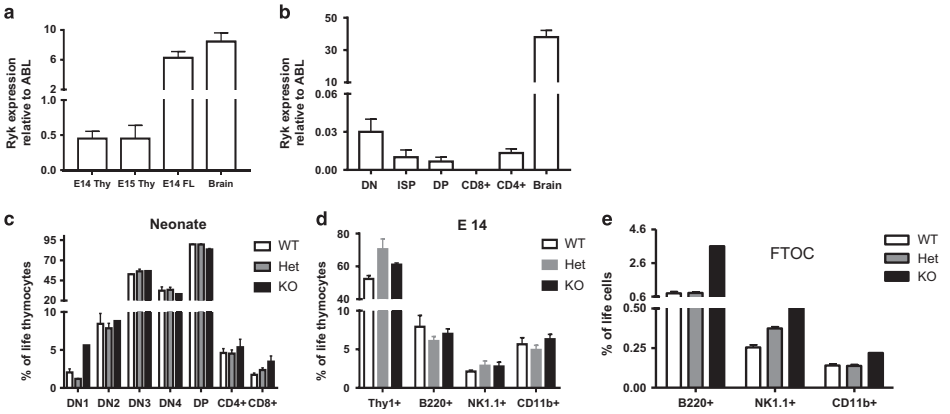

Figure 1. Gene expression analysis of Ryk in the murine hematopoietic system.

RT q-PCR analysis was performed to determine the level of Ryk expression normalized to ABL-2 expression as a housekeeping gene. The level of Ryk expression assessed in the thymic lobes and fetal liver E14 embryos (a) and adult T-cell developmental subsets in the thymus (b). Brain tissue was used as a positive control. Data are mean ± S.D. of six mice. Flow cytometric analysis performed in E14 thymocytes (c) and T-cell developmental subsets of neonates (d) in Ryk WT, Het, and KO mice. Data are mean ± S.D. of three mice per group (e) Frequency of non-T-cell lineages in cultured thymic lobes in fetal thymic organ cultures.

Because this spatially and developmentally regulated expression pattern suggested a potential functional the role for Ryk in thymopoiesis, we assessed thymic T-cell development phenotypically in Ryk-deficient neonates. However, no differences were observed in the T-cell developmental stages when we compared Ryk WT with Ryk/+ and Ryk −/− thymi in neonates (Figure 1c). We also looked into the thymocyte subsets in E14 thymic lobes and

again no difference was detected among different Ryk genotypes (Figure 1d). Yet, when thymic lobes were cultured *in vitro,* an increase in NK cells was observed.

It is known that Ryk can act as a co-receptor to induce Wnt signaling.[30] In fact, it has shown that Ryk can bind to both Wnt3a and Wnt5a, and trigger canonical and non-canonical Wnt signaling pathways, respectively.[24, 30, 31] To investigate the effects of Wnt signaling together with Ryk during T-cell development, we performed an *in vitro* T-cell development assay using E14 FL cells as a source of HSCs. In order to support multi-lineage differentiation, we mixed OP9 WT with OP9 DL1 (which induces T-cell development) in a 1:1 ratio as a control, and compared that with OP9 Wnt5a/DL1 1:1 and OP9 Wnt3a/DL1 1:1. T-cell development was assessed phenotypically at Day 7 and Day 14 post co-culture of FL cells with OP9 cells. No difference was observed between Ryk WT and Ryk KO FL cells (Figures 2a and b). We also examined NK cells, early B cells and myeloid cells but no significant phenotypic differences were detected between Ryk WT and Ryk KO FL cells in any of the conditions (data not shown).

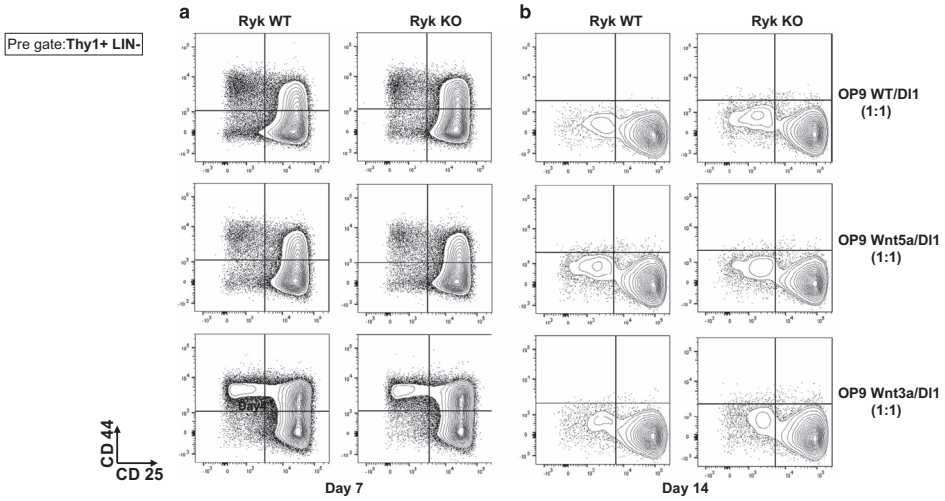

Figure.2 Phenotypic analysis of Ryk KOT cells developed in vitro in presence of prototype Wnt3a and Wnt5a.

E14 FL cells were obtained from Ryk WT and Ryk KO embryos and were co-cultured with mixture of OP9WT/DL1, OP9 Wnt3A/DL1, and OP9 Wnt5A/DL1 1:1. Cells were harvested 7 days (a) and 14 days (b) after co-culture and were analyzed flow cytometric for DN stages of T-cell development. The plots are pre-gated for Thy1+ and LIN− markers. The representative plots of two independent experiments are shown. Three mice per experiment were used.

In order to investigate the effect of Ryk deficiency during hematopoiesis and lymphopoiesis *in vivo* we performed competitive murine reconstitution assays. In such assays, the test and competitor population differ only in alleles of CD45 namely, CD45.1 (Ly5.1 historically) and CD45.2 (Ly5.2 historically). These two alleles are believed to be functionally identical, but can be readily discriminated by antibodies and allow for tracking of various cell populations and their progeny. LSK cells sorted from E14 FL Ryk WT or Ryk KO that bear the Ly5.2 congenic marker were mixed in a 1:1 ratio with WT LSKs from Ly5.1 congenic background. Next, the mixture of cells was transplanted into irradiated Ly5.1 recipients. A marked increase in CD3− NK1.1+ peripheral NK cells was observed in the recipients reconstituted by Ryk KO LSKs at week 7 post transplantation (Figure 3a). To follow-up on these observations, we analyzed the recipients every week. However, the phenotype was not observed at later time points (only at week 7 and 8 with a slight increase at week 9). At the end of the experiment we thoroughly examined NK cell development in the thymus, spleen and BM of the recipients and did not observe any differences between Ryk WT and Ryk KO recipients (Figure 3b). We also analyzed the stem cell reconstitution and LSK compartments in the BM of recipients. The Ly5.2 to Ly5.1 ratio of LSK (Figure 3c), LSK Flt3−, and LSK Flt3+ (Figure 3d) were not altered in the Ryk-deficient chimeric BMs. However, when thymic lobes were cultured *in vitro,* in a so-called fetal thymic organ culture, an increase in NK cells was observed consistent with the temporal increase in NK cells observed after transplantation. Hence, the mild phenotypic difference in NK cells may result from the mild block at DN stages, where NK cells in the thymus split off from the T-cell lineage.

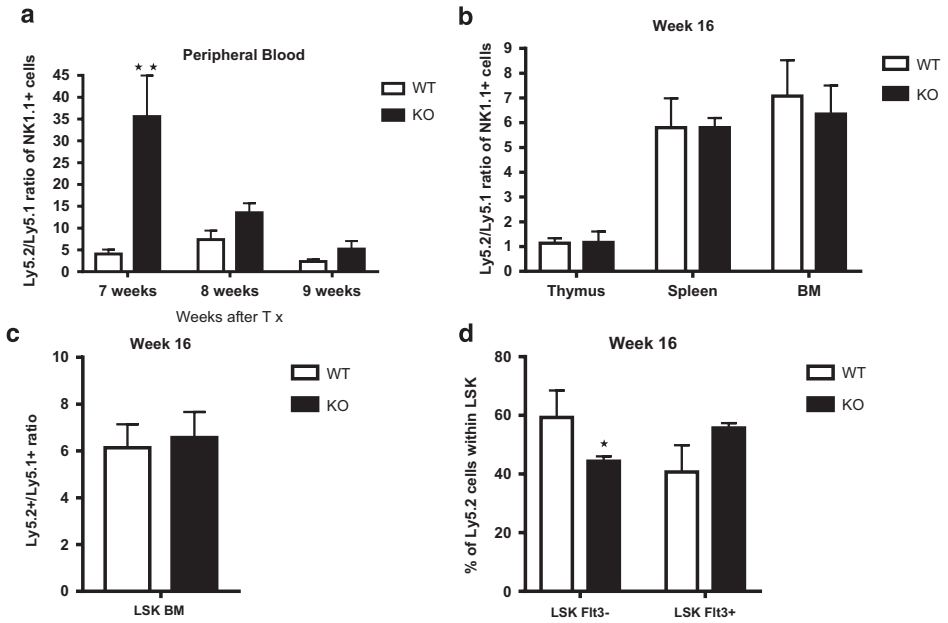

Figure 3. Peripheral NK cells analysis in recipient mice reconstituted by Ryk-deficient LSKs.

*Lin− Sca1+ Kit+ (LSK) cells were sorted from E14 FL Ryk WT and Ryk KO embryos and were transplanted intravenously into the Ly5.1-irradiated recipients. Peripheral blood analysis performed at 7, 8, and 9 weeks post transplantation. (a) The ratio of Ly5.2/Ly5.1 NK1.1+ CD3− cells in the recipient mice reconstituted with Ryk WT and Ryk KO LSKs is depicted. The recipients were killed 16 weeks after transplantation and the thymus, spleen, and BM were analyzed for NK cells. (b) The ratio of Ly5.2/Ly5.1 of NK1.1+ CD3− cells is depicted. The Ly5.2/Ly5.1 ratio of LSK compartments in the BM of recipient mice after 16 weeks of transplantation is shown (c and d). Data are mean ±S.D. of five mice per group. *P < 0.05 and **P < 0.01.*

Although, no significant alteration was observed owing to Ryk deficiency at the level of stem cells in BM, there seemed to be a trend of fewer stem cells in Ryk KO BMs (Figure 3d). As we detected high levels of Ryk gene expression in FL cells and most immature thymocytes, we hypothesized that Ryk might be involved in stem cell repopulation or self-renewal of HSCs. To further investigate this hypothesis, we performed secondary transplantation assay with chimeric BMs obtained from the primary recipients. This assay is the gold standard for investigating self-renewal properties of HSCs.[32] Three out of four mice reconstituted with Ryk KO primary chimeric BM showed lower reconstitution of Ly5.2+ cells compared with the recipients reconstituted with Ryk WT primary BMs (Figure 4a). Further analysis revealed that lower reconstitution in Ryk KO group was caused by fewer Ly5.2+ LSK cells relative to Ryk WT group (Figure 4b). In contrast to the stem cell-mediated phenotype, no significant differences were observed during T-cell developmental stages in the thymus (Figure 4c).

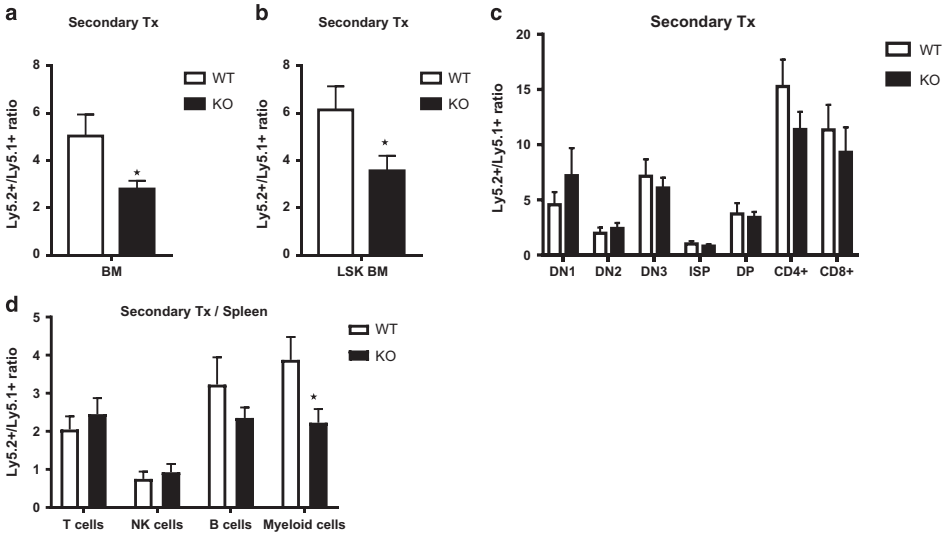

Figure 4. LSK and T-cell development analysis in secondary recipient.

*Primary BMs were obtained from the recipients reconstituted with Ryk WT and Ryk KO LSKs and transplanted into the Ly5.1-irradiated secondary recipient. Twelve weeks after transplantation, the secondary recipients were killed and BM and thymus were analyzed by flow cytometry. The ratio of Ly5.2/Ly5.1 (a) BM LSKs (b) and T-cell developmental subsets in the thymus (c) are depicted. Data are mean ±S.D. of four mice per group. *P<0.05.*

To study the mechanisms underlying reduced stem cell repopulation in the absence of Ryk, we performed apoptosis and proliferation analyses. E14 FL cells were obtained from Ryk WT and Ryk KO embryos, stained for LSK *ex vivo,* and the apoptosis and proliferation status of the cells was analyzed by flow cytometry. FL LSKs derived from Ryk KO embryos were more apoptotic showed by higher percentage of AnnexinV compared with the Ryk WT LSK cells (Figures 5a and c). However, the percentage of AnnexinV+ 7AAD+ dead cells were not altered in the Ryk KO LSK cells (Figures 5b and c). In addition, proliferation analysis of FL LSKs using the Ki67 marker revealed that the percentage of proliferative LSK cells is around two fold higher in Ryk KO FLs compared with the controls. (Figure 5d). This was confirmed using cell cycle analysis on FLs from Ryk KO and WT littermate controls (Table 1) showing that wild-type cells are much more in G1 (resting) than Ryk-deficient stem/progenitor cells, where almost twice as many cells are actively cycling indicating a loss of quiescence. Thus, a combined increase in apoptosis and proliferation explains the lower self-renewal of Ryk-deficient LSK cells.

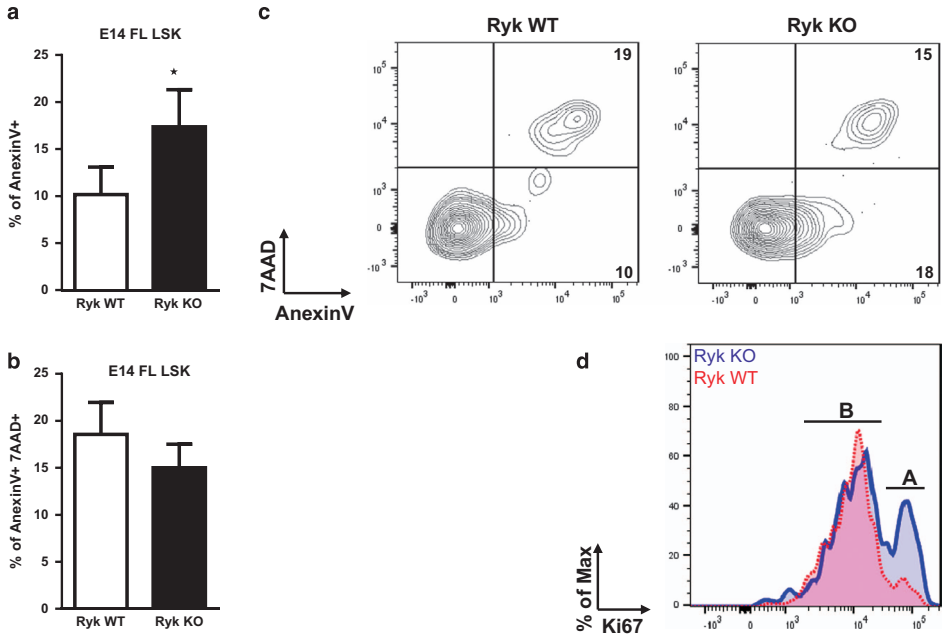

Figure 5. Apoptosis and proliferation analysis of E14 FL LSKs ex vivo

The percentage of AnexinV+ apoptotic cells (a) *and AnexinV+ 7AAD+ dead cells* (b) *of Ryk KO and Ryk WT E14 FL LSKs are depicted. The representative plot of three mice per group is shown* (c). *The proliferation status of cells was assessed by Ki67 staining. The representative plot of three mice per group is shown* (d). *Data are mean ±S.D. of three mice per group. *P<0.05.*

Table 1 Cell cycle analysis of Ryk-deficient vs wild-type fetal liver stem/progenitor cells

Genotype	*G1 (%)*	*S (%)*	*G2/M (%)*	*Resting/cycling*
wt	41	48	12	0.68
wt	39	48	15	0.62
Ryk – / –	23	56	20	0.30
Ryk – / –	22	60	14	0.29

Discussion

In this study we used a full Ryk loss-of-function model for the first time to investigate the role of this non-canonical Wnt receptor during hematopoiesis and lymphopoiesis. The model represents the null allele of Ryk generated by homologous recombination in which 14.5 kb of genomic DNA including exons encoding >95% of the Ryk extracellular domain and the entire transmembrane domain are deleted.[28, 33] Our data suggest that Ryk as a co-receptor has a marginal role in hematopoiesis, possibly owing to a redundant role with other tyrosine kinase receptors including Ror,[34, 35] or Wnt signaling receptors such as FRZ 2,[30, 36]and or FRZ8 in combination with Flamingo.[37] During neurogenesis, Ryk's function is vital, which is consistent with its high level of gene expression in neural tissues.[36] The importance of Ryk during embryogenesis is well studied.[23 38] Indeed, we also observed that in hematopoietic tissues the level of Ryk expression is higher in FL or fetal thymic lobes compared with the adult tissues (Figure 1).

It has previously been shown that Ryk's function, similar to other receptors, is context and tissue dependent.[23, 34] Most probably, this is determined by several factors, including abundancy of specific triggers in various tissues, or the expression of distinct co-receptors by neighboring cells. Ryk can bind to both the Wnt3a canonical ligand,[24] and Wnt5a non-canonical ligand[26] depending on the context and type of tissue, suggesting that different experimental settings might result in different outcomes. Our data do not support an important specific role for Ryk during T-cell development as no differences between mutant and control were observed *in vitro* in presence of both the Wnt3a and Wnt5a ligands.

We showed that Ryk has a role in stem cell repopulation when we performed secondary transplantations. Ryk KO stem cells undergo more apoptosis and are more proliferative compared with wild-type cells. Nemeth and co-workers have proposed that Ryk, by binding Wnt5a, can suppress proliferation of LSK cells.[25, 39] In these studies on Ryk's function in hematopoiesis, anti-Ryk polyclonal antibodies that presumably block the receptor have been employed.[24, 26] These investigators showed that by adding polyclonal antibodies to the Ryk receptor a modest decrease in cells in G0 (from 29 to 22%) was observed, which was interpreted as a loss-of-function effect, in which blocking Ryk would increase proliferation; this in line with our observation on genetically deficient Ryk stem cells. However, polyclonal antibodies have variable effects and could include both stimulating and inhibitory antibodies. In addition, structural and functional studies have shown high levels of redundancy between Ryk and other members of tyrosine kinase receptors, in particular the ROR non-canonical Wnt receptors, making the study of each receptor specifically cumbersome.[34] Given these structural similarities, it is even possible that an anti-Ryk polyclonal antibody could cross react with other non-canonical receptors. Thus,

it is uncertain if all effects attributed to blocking Ryk, could be assigned to Ryk, Ror or perhaps other receptors. A clear loss-of-function model as we employed here allows a more direct interpretation of the role of Ryk in hematopoiesis, although molecular redundancy by the related Ror receptors could also play a role here. The effects of treatment with polyclonal Ryk antibodies on long-term hematopoietic reconstitution were similar to ours results, that is, lower reconstitution when Ryk's function was lost. We have chosen to use competitive transplantation, as this reduces mouse to mouse variability and possible effects of an antibody treatment on non-hematopoietic cells can be excluded, for instance on niche cells that express Ryk. Using this system, we here provide definitive proof for Ryk's functional role in HSC self-renewal via competitive secondary transplantation, the gold standard assay to assess HSC self-renewal. Thus, Ryk deficiency in HSCs reduces their quiescence, leading to proliferation-induced apoptosis and decreased self-renewal.

Our data also suggest that a timing–dependent role for Ryk in hematopoietic tissues. We observed that peripheral NK cells are temporary higher in initial assays of recipients transplanted with Ryk KO FL cells compared with wild-type group. One possible explanation is that Ryk is only important in a certain stage of NK cell development, as a default pathway during thymic T-cell development when TCR rearrangements cannot successfully be accomplished and multipotent cells choose a NK cell fate. As the cells pass that specific stage and a critical number of T cells have been generated, the role of Ryk would become less important.

Concluding, besides a developmental window of time for NK lineage development, the effects of Ryk on thymopoiesis are apparently limited. The combined increases in apoptosis and loss of quiescence in HSCs, likely underlie the lower self-renewal of Ryk-deficient LSK cells. The roles of canonical and non-canonical Wnts and potential cross-talk between the pathways, clearly require more research, particularly, as Wnts are being employed in stem cell expansion protocols,[40, 41] including those employing designer nucleases for therapeutic gene editing.[42] Finally, increasing evidence indicates the involvement of both canonical and non-canonical Wnts in hematological malignancies (e.g., reviewed in ref. 13). As we showed previously, the dosage of canonical Wnt signaling is critical in determining the functional outcome on hematopoietic cells[43, 44] and investigating Wnt proteins for HSC expansion will require good *in vivo* reporter systems and well-characterized reagents to take effects on apoptosis as well as on cell proliferation into account. Collectively, such tools would help capitalizing on the inherent power of the canonical and non-canonical Wnt pathway to regulate apoptosis and self-renewal of stem cells.

Materials and Methods

Mice

Mice were bred and maintained in the animal facilities of Leiden University Medical Centre, in accordance with legal regulations in The Netherlands and with the approval of the Dutch animal ethical committee. C57Bl/6-CD45.1 (Ly5.1) and C57Bl/6-CD45.2 (Ly5.2) mice were obtained from the Jackson Laboratory, and Ryk KO mice were kindly provided by Dr. Stacker.[28]

Flow cytometry

The following antibodies were obtained from BD Biosciences (San Diego, CA, USA): anti-CD3-APC (145-2C11), anti-cKit-Pe-Cy7 (2B8), and anti CD11b-PE (M1/70). For Lineage depletion these markers were used: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), Gr1 (RB6-8C5), B220 (Ra3-6B2), Ter119 (Ly76) and Nk1.1 (PK136) biotin and subsequently were stained with streptavidin eFluor 450 (48-4317) from eBioscience (Vienna, Austria). The following antibodies were also purchased from eBioscience: Ly5.1-PE-Cy7 (A20), Ly5.2 Alexa Fluor 780 (104), B220 PE-Cy7 (RA3-6B2), Gr1 eFluor 450 (RB6-8C5) and Sca1 PE-Cy7 (D7). Cells were stained in fluorescence activated cell sorter (FACS) buffer (PBS, 2% bovine serum albumin, 0.1% sodium azide) for 30 min at 4 °C. Ultimately, cells were washed and measured either on a Canto I, or an Aria (BD Biosciences) FACS. For FL LSK, Mac1 was precluded from the lineage gate, as FL LSK express Mac1.[45] For apoptosis analysis, E14 FL cells were stained with 7AAD/AnnexinV kit (BD Bioscience) in combination with LSK staining. For proliferation analysis E14 FL cells were stained with PE mouse anti-Ki67 set (BD Pharmingen, San Diego, CA, USA) in combination with LSK staining or for cell cycle analysis with an adapted protocol for combined LSK and propidium iodide staining.[46] Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Co-culture of FL cells with OP9 cell lines and fetal thymic organ cultures (FTOC)

In total, 50 000 total FL cells were obtained from Ryk WT and Ryk KO mice and were co-cultured on confluent layers of OP9 WT/DL1, OP9 Wnt3a/DL1, or OP9 Wnt5a/DL1 mixed in a 1:1 ratio as described previously[27] with Alpha MEM 10% FCS containing 50 ng/ml rmSCF, 10 ng/ml rmFlt3L and 10 ng/ml rmIL-7 (all cytokines from R&D Systems, Abinsdon, UK) in 24-well plates. Cells were harvested after 7 and 14 days of co-culture and assessed for T-cell development by flow cytometric analysis. FTOC were done as described before[47] using fetal thymic lobes from E14 embryos, which were genotyped for the status of the Ryk deficiency. Thymic lobes were cultured on a nitrocellulose filter on air/medium interphase for 7–14 days, dispersed, and analyzed by flow cytometry.

Ryk gene expression analysis

Total RNA was extracted using Qiagen RNeasy mini or micro columns. One mirogram of total RNA was used as a template for cDNA synthesis, using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), Oligo dT, and random hexamer primers. The RT-PCR reaction was performed using TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA, USA) and was run on a PRISM 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). The following primers were used in combination with FAM-labeled probes from the universal probe library (Roche, Almere, Netherlands): mouse Ryk forward primer: 5′-CAAGCTTCGAGGTCTGCAC-3′ reverse primer: 5′-ACCATGGGCTTTTCTCCTTC-3′. RQ-PCR results were normalized to Abl expression in the same sample: forward primer: 5′-TG-GAGATAACACTCTAAGCATAACTAAAGGT-3′ reverse primer: 5′-GATGTAGTTGCTTGGGACCCA-3′ and probe: 5′-FAM-CCATTTTTGGTTTGGGCTTCACACCATT- NFQ-3′.

Competitive transplantation assay

Primary transplantation assays were performed with the Ly5.1/Ly5.2 system. LSK cells were sorted from Ryk WT and Ryk KO (Ly5.2 background) and Ly5.1 WT FLs. In total, 2×10^3 Ryk WT or Ryk KO LSKS were mixed 1:1 with Ly5.1 WT LSKs, and were transplanted intravenously into lethally irradiated (8 Gy) Ly5.1 (9–12 weeks) mice together with 3×10^5 Ly5.1 splenic support cells. Chimeras were analyzed at 4, 8, and 12 weeks after transplantation in peripheral blood, and mice were killed for analysis at 16 weeks post transplantation except where otherwise indicated. Mice were considered repopulated when>1% multi-lineage Ly5.2 cells could be detected in nucleated peripheral blood cells 3 months after transplantation. For secondary transplantation, equal numbers of total BM cells from primary recipients that received Ryk WT or Ryk KO LSKs were pooled and transplanted into lethally irradiated Ly5.1 secondary recipients. Peripheral blood from secondary transplanted mice was analyzed at 4, 8, and 12 weeks post transplantation. Thirteen weeks after transplantation mice were killed and BM, thymus, and spleen were analyzed.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test (Prism GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Acknowledgments

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CHAPTER 4

Discrete roles of canonical and non-canonical Wnt signaling in hematopoiesis and lymphopoiesis

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Abstract

The mechanisms that regulate proliferation, fate decisions and differentiation of hematopoietic stem cells (HSC) and thymic stem cells are highly complex. Several signaling pathways including Wnt signaling have important roles during these processes. Both canonical and non-canonical Wnt signaling are important in normal and malignant hematopoiesis and lymphoid development, yet their precise roles are controversial. In a side-by-side comparison, we investigated the roles of the canonical and non-canonical Wnt pathway in hematopoiesis and thymopoiesis. As complete loss-of-function models for non-canonical Wnt signaling are not yet available and highly complex for canonical Wnt signaling, we decided to use a gain-of-function approach. To this end, Wnt3a and Wn5a, two well-known prototypical canonical and non-canonical Wnt ligands were produced in hematopoiesis supporting stromal assays. High levels of Wnt3a signaling blocked T-cell development at early stages, whereas intermediate levels accelerated T-cell development. In contrast, Wnt5a signaling prompted apoptosis in developing thymocytes, without affecting differentiation at a particular stage. To explore the role of Wnt3a and Wnt5a *in vivo*, we transduced HSCs isolated from fetal liver, transduced with Wnt3a and Wnt5a vectors, and performed reconstitution assays in irradiated C57Bl/6 mice. Wnt3a overexpression led to increased lymphopoiesis, whereas Wnt5a augments myelopoiesis in the bone marrow (BM) and spleen. Thus, the canonical and non-canonical Wnt signaling have discrete roles in hematopoiesis and thymopoiesis, and understanding their right dose of action is crucial for prospective translational applications.

Introduction

The development of blood and immune cells are highly complex and regulated processes. A wide variety of signaling pathways has been implicated in these processes. Several developmental signals have key roles in both the bone marrow (BM) and thymus, such as BMP, Wnt and Notch signaling.[1, 2] Hematopoietic stem cells (HSC) are rare BM-residing cells with the capacity to self-renew and differentiate into all blood cell lineages. All blood cells, except T lymphocytes, develop within the BM. Different types of progenitor cells migrate from BM to thymus where they develop to mature T cells.[3, 4] The nature of these cells is still subject of debate. As only few early thymic progenitors (ETPs) arrive in the thymus (<10/day), massive proliferation is necessary to establish a pool of T-cell progenitors.[5] During development, these immature thymocytes gradually lose their proliferative and multilineage potential, and initiate a T-cell developmental program, a process termed T-cell commitment. Notch signaling has been shown to have an important role during T-cell commitment by directly or indirectly upregulation of T-cell-specific genes including *Ptcra*, *Cd3e* and *Zap 70*.[6, 7] Other soluble factors including Wnt ligands might also be crucial for T-cell proliferation and commitment.[8, 9, 10]

Early stages of T-cell development are phenotypically characterized by absence of the mature T-cell markers CD4 and CD8 and referred to as double negative (DN).[11] DN stages are subdivided into four stages. DN1: CD44+ CD25–, DN2: CD44+ CD25+, DN3: CD44− CD25+ and DN4: CD44– CD25–.[8, 12] It is believed that T-cell commitment occurs at the transition of DN2 to DN3 stages.[13, 14] Afterwards, thymocytes develop to the immature single positive (ISP) stage defined as CD3− CD8+. Thymocytes with functionally rearranged T-cell receptors (TCRs) develop to next stage, which is double positive (DP) for CD4 and CD8 and finally they become mature single positives (SP)[12] either CD4 or CD8.[7, 8, 9]

Thymic epithelial cells (TECs) provide a unique environment for ETPs to develop towards T cells.[15, 16]TECs also express high levels of Notch ligands including Delta like ligands 1 and 4, soluble Wnt ligands and IL-7, which all are crucial for early stages of T-cell development.[17]

The Wnt signaling pathway is subdivided into canonical (*β*-catenin dependent) and non-canonical (*β*-catenin independent) pathways. Binding of different Wnt proteins to frizzled (Fzd) receptors can trigger different Wnt pathways. The diversity of ligands and receptors makes the study of Wnt signaling from point of view of cell surface receptors and ligands challenging. Wnt proteins function as proliferation-inducing growth factors but may also affect cell-fate decisions, apoptosis and quiescence.[18] Canonical Wnt proteins bind to their receptors, thereby preventing proteosomal degradation of the Wnt-mediator *β* -catenin. Subsequently, *β* -catenin trans locates to the nucleus where it will form an active transcrip-

tion complex with one of the four transcription factors downstream of the Wnt pathway: Tcf1, 3 or 4 (T-cell Factor 1, 3, 4) or Lef1 (lymphocyte-enhancer-binding factor). Upon transcriptional activation, several target genes will be activated including *Axin2, c-fos, c-myc* and many others, which are important for proliferation and/or cell-fate decisions. Non-canonical Wnt signaling involves recognition of distinct Wnt ligands by a cognate Frz-LRP receptor complex, heterotrimeric G protein activation of phospholipase C as well as the release of intracellular Ca^{2+} ions. Non-canonical Wnt signaling also regulates cellular polarization and migration (the so-called planar-cell-polarity pathway).[9, 10, 18, 19, 20, 21]

A large body of evidence has shown the significance of canonical Wnt signaling during T-cell development. Generation of Tcf1 KO mice provided the first evidence of a Wnt signaling effect during T-cell development.[22] Tcf1 deficiency partially blocks T-cell development at various early DN stages, resulting in fewer mature T cells and smaller thymus. In addition, Tcf1/Lef double KO mice have a complete block at the ISP stage, which indicates redundancy between these factors during thymocyte development.[23]

Similar to Tcf1 deficiency, fetal thymic organ cultures using Wnt3a-deficient progenitors exhibited progressively impaired T-cell development caused to an ISP block.[24] Several loss-of-function and gain-of-function studies have targeted the core Wnt-mediator *β*-catenin in thymocytes. Conditional *β*-catenin deletion using Lck-Cre impaired *β*-selection of TCR,[25] whereas *β*-catenin overexpression regulates positive selection and generation of SP CD4/ CD8.[26] Inhibiting the interaction between *β*-catenin and Tcf1 also blocks the DN to DP transition.[27] Wnt signaling is active at various stages of T-cell development but most predominantly at DN stages. Indeed, inhibiting Wnt signaling by using Dickkopf (DKK1) as Wnt sequestering molecule, blocks the development at the most immature DN1 stage.[21] Finally, genetic proof that canonical Wnt signaling is crucial for normal T-cell development stems from complementation studies in which only the large form of Tcf1 that can interact with *β*-catenin and transduce Wnt signals was capable to restore T-cell development, whereas the short form that lack the *β*-catenin domain could not.[28]

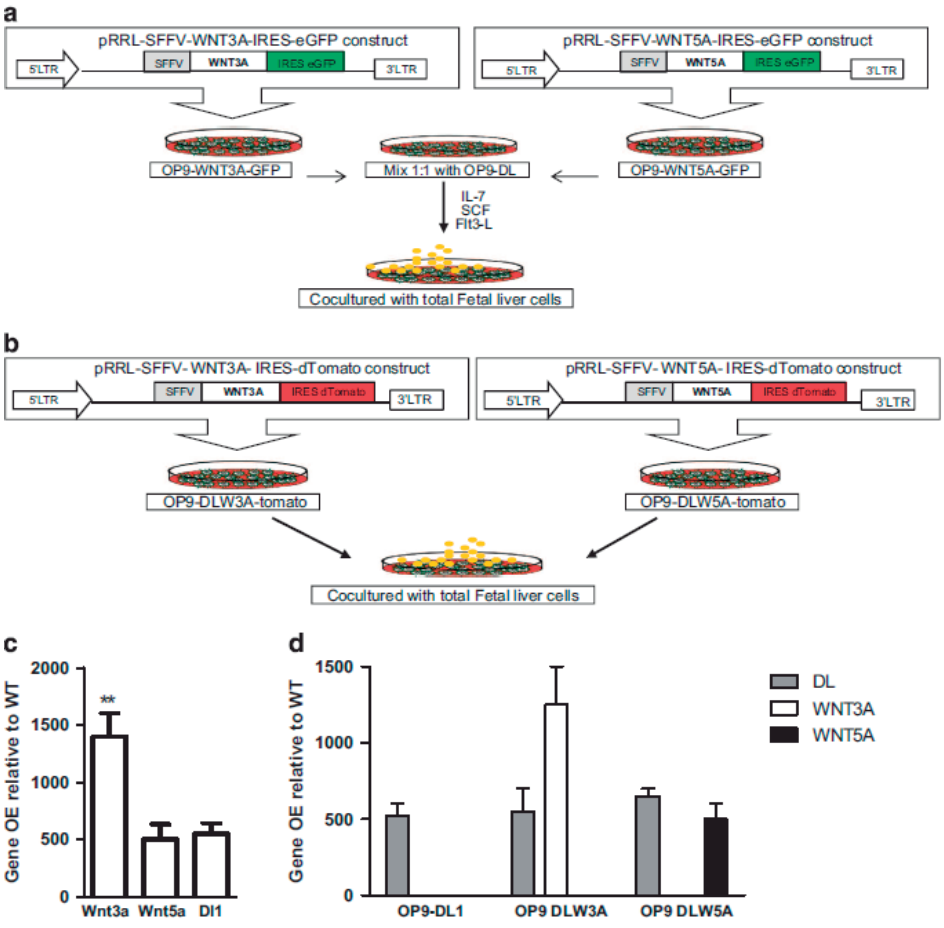
On contrary, there are only a handful of studies focused on the role of non-canonical Wnt signaling in T-cell development. Liang *et al.*[29] showed that Wnt5a deficiency protects against apoptosis in DP stage, but it was proposed to be irrelevant at early stages of T-cell development. Another series of studies revealed that Wnt4 induces expansion of Lin- Sca1+ Kit+ (LSKs) in BM, which subsequently causes ETP expansion in thymus.[30] Wnt4 regulates ETP expansion via a TEC-dependent mechanism.[31] Whether Wnt4 functions as a canonical or non-canonical ligand is still debatable, in particular in gain-of-function studies in which thymopoiesis was increased. Perreault and colleagues showed that Wnt4 binds to Fzd6 and activates JNK kinase via PCP pathway,[32] strongly suggesting that Wnt4 signals in a non-canonical fashion.

Loss-of-function approaches using *β*-catenin and *γ*-catenin have often not revealed thymic phenotypes,[33]most likely because Wnt signaling is still present at appreciably levels in these models.[34] In addition, during T-cell development canonical and non-canonical Wnt signaling have only been studied in isolation but not together. Here, we aimed to side-by-side compare the two prototypical canonical and non-canonical Wnt ligands, that is, Wnt3a (canonical) and Wnt5a (non-canonical).

Results

OP9-based in vitro assays for T-cell development

To study the role of canonical and non-canonical Wnt signaling during T-cell development *in vitro*, we generated two different OP9-based assays. First, we transduced OP9-WT cell line with Wnt3a and Wn5a constructs linked to an IRES-GFP cassette, mixed the transduced cell lines with the OP9-Dl1 cell line in 1:1 ratio to support T-cell development (Figure 1a). Initially, we examined efficiency of the OP9 cell mixtures for support of T-cell development in comparison with OP9-Dl1 cell line alone. The aim of mixing Wnt3a- and Wnt5a-producing OP9 cells with OP9-DL11 was (a) supporting T-cell development in contrasting situation of abundant Wnt3a vs Wnt5a (b) creating an culture system to study T and B lymphoid as well as myeloid development simultaneously. Although there was a slight delay in T-cell development at 14–21 days, the mixture could also efficiently support T-cell development up to the DP stages, whereas OP9-WT did not induce T-cell development (See Supplementary Figure 1).



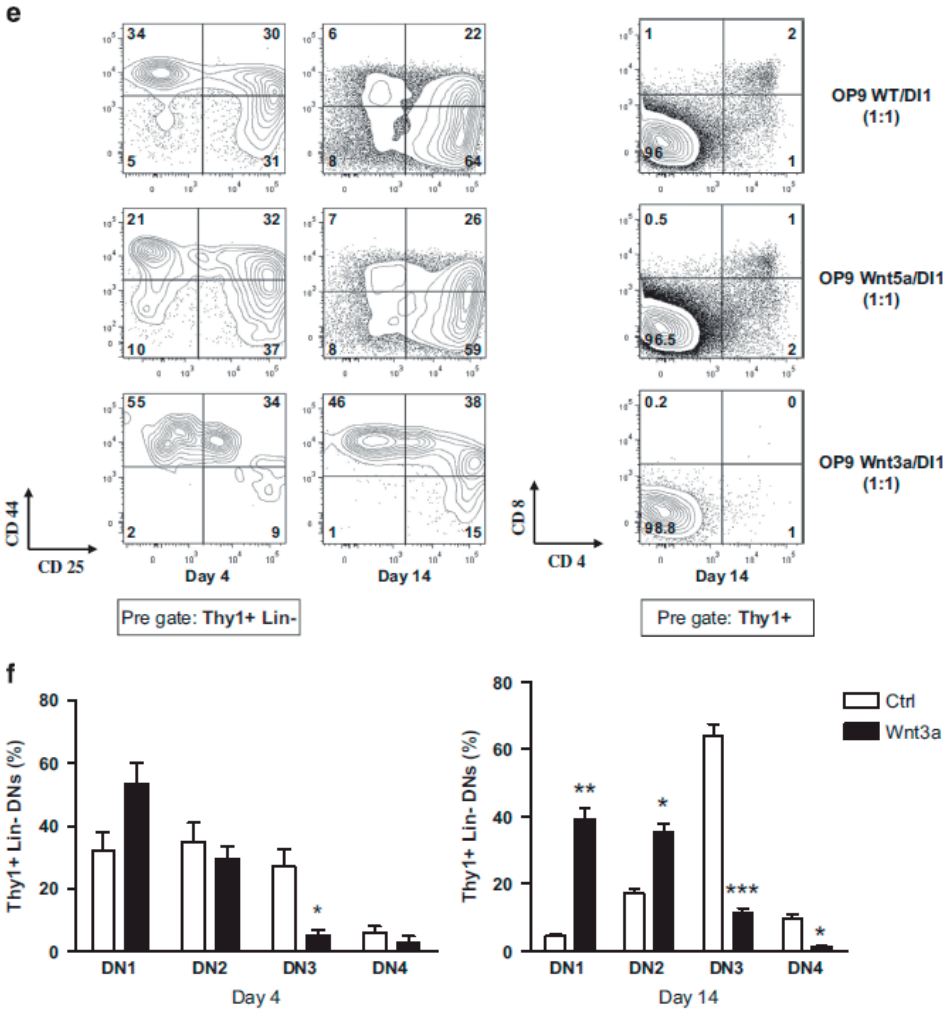


Figure 1. Canonical Wnt3a overexpression blocks T-cell development at early stages.
*(**a**) Experimental design. OP9-WT cell lines were transduced with pRRL-SFFV-Wnt3a-GFP or pRRL-SFFV-Wnt5a-GFP lentiviral constructs, selected by cell sorting on high GFP expression and for experiments mixed with OP9-Dl1-GFP. Stem cells from E14 fetal liver cells were co-cultured with OP9-WT/Dl1 mixed 1:1 as a control, or OP9-Wnt3a/Dl1 1:1, or OP9-Wnt5a/Dl1 1:1 for 14–21 days in aMEM 10% FCS in presence of IL-7, SCF and Flt3L cytokines. (b) Experimental design. OP9-Dl1-GFP cell lines were transduced with pRRL-SFFV-Wnt3a-tomato (DLW3A) or pRRL-SFFV-Wnt5a-Tomato (DLW5A) and selected by cell sorting on high GFP expression. Stem cells from E14 fetal liver cells were co-cultured with OP9-Dl1 as a control, OP9-DLW3A or OP9-DLW5A for 14 days in aMEM 10% FCS in presence of same cytokines. (c) RTq-PCR analysis was performed to determine overexpression of Wnt3a (left bar), Wnt5a (middle bar) and Dl1 gene (right bar) in transduced OP9 cell lines. The levels of expression are normalized by ABL-2 expression and presented as fold induction relative to untransduced OP9 cell line.*

***Figure 1. Canonical Wnt3a overexpression blocks T-cell development at early stages**. (continued)*

*(d) The overexpression of Dl1 gene (gray bars), Wnt3a gene (white bars) and Wnt5a gene (black bars) in untransduced OP9DL1 cell line (left), OP9-DLW3A cell line (middle) and OP9-DLW5A (right) are shown. The levels of expression are normalized by ABL-2 expression and presented as fold induction relative to untransduced OP9 cell line. (e) Total fetal liver cells were co-cultured with OP9-WT/DL1:1 as control (top row), OP9-Wnt5a/DL1:1 (middle row) or OP9-Wnt3a/DL1:1 (bottom row). Cells were harvested 4 days and 14 days after co-culture and were analyzed flow cytometric for DN stages of T-cell development. The plots are pre-gated for Thy1+ and LIN- markers. Lineage markers include CD3e, CD4, CD8a, CD11b, Gr1, B220, NK1.1 and Ter119. The percentage of each population is indicated. (f) Collective data of total experiments from (e) are depicted. Total fetal liver cells are co-cultured with OP9-WT/DL1:1 as control (white bars) or with OP9-Wnt3a/DL11:1 (Black bars). Cells were harvested at day 4 and day 14 of co-culture and were assessed by FACS for DN stages of T-cell development. The percentage of each stage is shown within Thy1+ Lin– population. Data are mean ±S.D. of nine control and eight Wnt3a samples from three independent experiments. *P<0.05; **P<0.01; ***P<0.001*

We also generated another OP9-based assay in which we directly transduced OP9- Dl1-GFP cell line with Wnt3a and Wnt5a-tomato constructs, and assessed *in vitro* T-cell development (Figure 1b). We quantified relative expression of Wnt3a, Wnt5a and Dl1 using Q-PCR. Wnt3a had over 1000-fold higher expression compared with OP9-WT, whereas Wnt5a had ~500-fold and Dl1 remained to be expressed at levels over 700-fold (Figure 1c). In OP9-DLWnt3a-tomato (DLW3A) and OP9-DLWnt5a-tomato (DLW5A) Wnt3a and Wnt5a were expressed as high as in the OP9-Wnt3a-GFP and OP9-Wnt5a-GFP, respectively, and Dl1 expression was not altered compared with the OP9-Dl1-GFP (Figure 1d).

Canonical Wnt3a overexpression blocks T-cell development at early stages, thereby favoring development of alternative lineages

We cultured fetal liver cells (as these provide a good source of both T-cell progenitors and stem cells) either with the mixture of OP9-WT/Dl1 in 1:1 ratio as a control, or with OP9-Wnt3a/Dl1 or OP9-Wnt5a/Dl1 with the same ratio for 14 days. Wnt3a overexpression blocked T-cell development at the DN1 stage (Figures 1e and f).

The mixture of OP9-WT and OP9-Dl1 provides a means to study development of B, T, NK and myeloid lineages simultaneously.[35] DN1 and DN2 immature thymocytes have the potential to develop towards other lineages. Consistently, in the Wnt3a-overexpressing cultures, non T cells, such as B and myeloid cells developed more efficiently compared with the control (Figures 2a and b). The difference was not only observed in proportion of each lineage, but also in the absolute numbers (Figure 2c).

Similar data were observed when Wnt3a and Wnt5a were expressed in the OP9-Dl1 cells themselves (supplementary Figure 2). Therefore, we conclude that canonical Wnt3a signaling has the capacity to inhibit T-cell development at early DN2-DN3 stages, and to

increase alternative (myeloid and B cell) development. Wnt5a overexpression in neither of assays did show any phenotypic difference regarding T-cell development (data not shown).

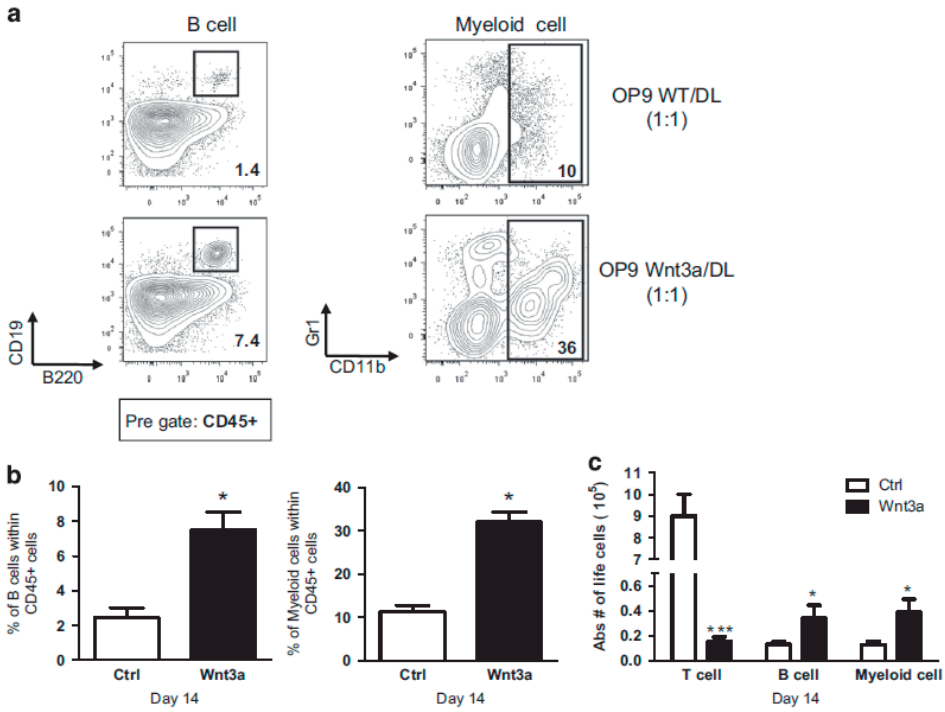


Figure 2. Wnt3a overexpression favors alternative lineages development.

*(a) Total fetal liver cells were co-cultured with OP9-WT/DL1:1 (top row) or with OP9-Wnt3a/DL11:1 (bottom row). Cells were harvested after 14 days of co-culture and were analyzed by FACS for B cells (B220 and CD19) and myeloid cells (CD11b and Gr1). The plots are pre-gated on CD45+ cells. Representative plots of two independent experiments are shown. (b) Collective data of all experiments are depicted. OP9-WT/DL (Ctrl) are depicted in white bars and OP9-Wn3a/Dl (Wnt3a) in black bars. The left bar graphs present percentage of B cells (B220+ CD19+) within the CD45+ gate, and right bar graphs present percentage of myeloid cells (CD11b+ Gr1+) within the CD45+ g ate. (c) Absolute number of total CD45+ life cells after 14 days of co-culture are depicted. Data are mean ±S.D. of six controls and five Wnt3a samples from two independent experiments. *P<0.05; **P<0.01; ***P<0.001*

Wnt5a overexpression results in increased apoptosis in developing thymocytes, whereas Wnt3a does not

Further analysis revealed that total percentage of lymphocytes was around fourfold lower in the Wnt3a-overexpressing group at various time points after co-culture (4, 7 and 14 days) (Figure 3a). This could be caused by increased apoptosis in the Wnt3a-expressing cultures. We performed apoptosis assays by using AnexinV and 7AAD in combination with the Thy1 marker to separate T cells from non-T cells. In the Wnt3a-overexpressing cultures total

Thy1+thymocytes and specifically DN thymocytes were not undergoing any a significant level of apoptosis (Figure 3a). There is an increase in apoptosis in non T cells, yet not to the extent that non T cells would decrease in numbers in comparison with thymocytes (see Figures 2b and c). Thus, we concluded that inhibition of differentiation toward T lineage due to Wnt3a overexpression causes low cellularity in this culture.

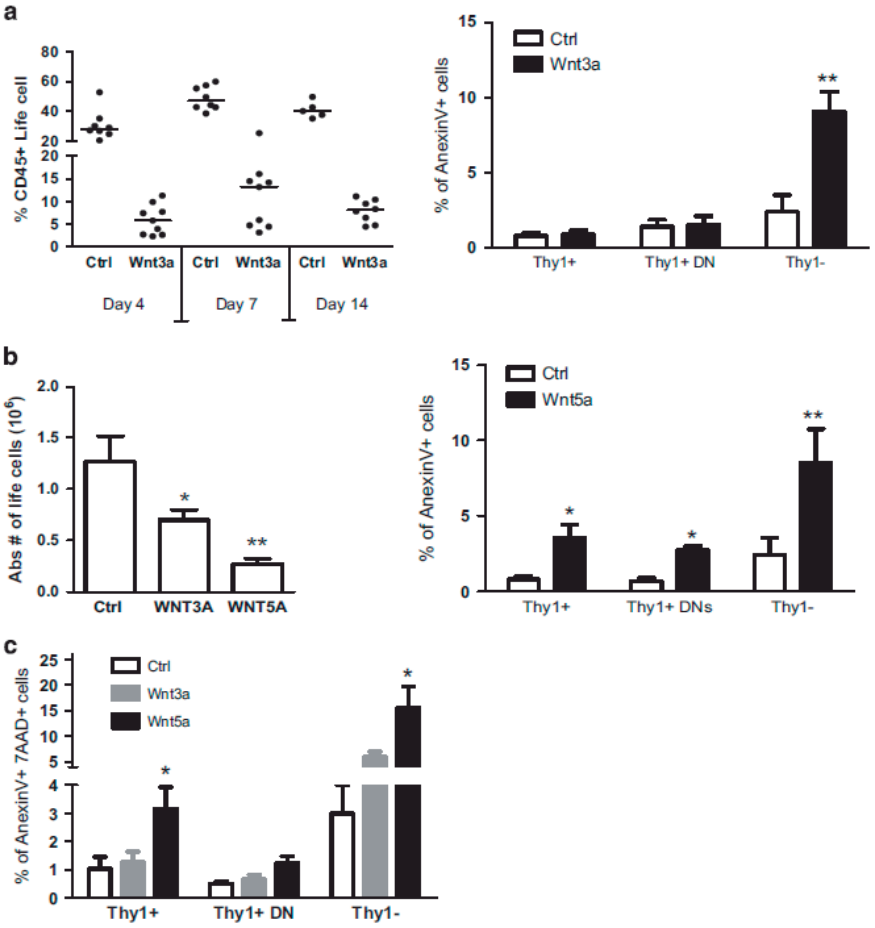


Figure 3. Wnt5a overexpression results in apoptosis in developing thymocytes.

(a) Total fetal liver cells were co-cultured with OP9-WT/DL1:1 (Ctrl) or with OP9-Wnt3a/DL1:1 (Wnt3a) or with OP Wnt5a/Dl1 (Wnt5a). In the left graph, the percentage of total CD45+ life cells were assessed by FACS after 4 days, 7 days and 14 days of co-culture. The averages are indicated by a dash. Each dot represents one mouse. In the right graph, the percentage of AnexinV+ apoptotic cells is shown in specified populations after 14 days of co-culture with OP9-WT/DL (white bars) or with OP9-Wnt3a/DL (black bars). Error bars represent mean±S.D. from two independent experiments (each in triplicates). (b) In the left graph, the absolute number of life cells is shown after 14 days of co-culture. Error bars represent mean±S.D. from two independent experiments (each in triplicates). In the right graph, the percentage of AnexinV+ apoptotic cells is shown in specified populations after 14 days of co-

*culture with OP9-WT/DL (white bars) or with OP9-Wnt5a/DL (black bars). Error bars represent mean±S.D. from two independent experiments each in triplicates. (c) Percentage of AnexinV+AAD7+ cells representing dead cells in the culture gated on tall thymocytes, DN thymocytes and non-T lineage cells in thymic cultures with Wnt3a or Wnt5a expressed in OP9-DL1 cells. *P<0.05; **P<0.01; ***P<0.001.*

Although the Wnt5a-overexpressing cultures did not show any phenotypic differences compared with the controls, the absolute numbers were around three- to fivefold lower (Figure 3b). Lack of Wnt5a promotes Bcl-2 expression and inhibits apoptosis of DP thymocytes.[29] Our analysis showed that both Thy1+ and Thy1– co-cultured cells were undergoing more apoptosis. Thus, this gain-of-function approach showing more apoptosis is consistent with the loss-of-function Wnt5a experiments reported before. The effect of apoptosis in thymocytes (DN and Thy1+) by Wnt5a was much stronger than Wnt3a, which was similar to controls. Interestingly, for non T cells, similarly high apoptosis was found. The strong effects of Wnt5a on cell death are also reflected in the strong increase in 7AAD+AnnexinV+ DP cells (Figure 3c).

Optimal dosage of Wnt3a signaling accelerates T-cell development

Gene expression analysis revealed that Wnt3a was very highly expressed in our transduced OP9 cells (Figures 1c and d). This is 500-fold higher than the physiological level, for example, as found in fetal thymus,[21] which we used as comparison. We have previously shown that canonical Wnt signaling functions in a dosage-dependent fashion during HSC reconstitution and T-cell development using Apc hypomorphic models.[19] We hypothesized that the same would hold true with the Wnt3a gain-of-function model. We modified the Wnt3a concentration in the culture by serially mixing OP9-DL with OP9-DLW3a at various ratios.

After 14 days of co-culture, the majority of cells developed into DN3 and DN4 with 32% of DN4 thymocytes. Interestingly, the co-cultured cells with 1% DLW3A (which has fivefold higher overexpression relative to the physiological level, supplementary Figure 3), showed an accelerated T-cell development with 50% DN4 thymocytes development. However, T-cell development was inhibited again by increasing the concentration of DLW3A (10% and 50% Wnt3a), and was completely blocked at the DN3 stage with 100% OP9-DLW3A alone (Figure 4a).

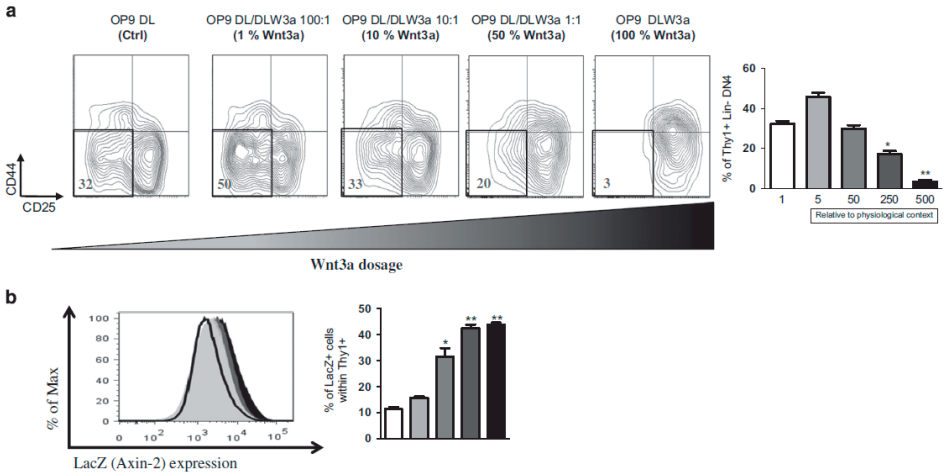


Figure 4. Optimal dosage of canonical Wnt signaling accelerates T-cell development.

*(a) Total FL cells were co-cultured for 14 days with OP9-DL1, or different ratios of OP9-DLW3A/ OP9-Dl1 mixture as indicated. Cells were then harvested and were analyzed by FACS for DN stages of T-cell development. The percentage of CD44− CD25− DN4 is indicated. The plots are pre-gated on Thy1+ LIN− markers. Error bars represent mean ±S.D. of one independent experiment in triplicates. Below the bar graphs Wnt3a gene overexpression relative to the physiological levels are indicated. (b) Total FL cells from Axin2 LacZ (wnt reporter mice) were co-cultured with OP9-DL1 and different ratios of OP9-DLW3A as previously indicated. Cells were harvested and β-galactosidase (LacZ) activity in Thy1+ cells was measured. Quantification of the mean fluorescence intensity (MFI) of Thy1+ cells is shown (left). Quantification of the frequency of LacZ+ cells is shown (right). Littermate mice not carrying the reporter transgene (Axin2 +/+) were used to define the LacZ− population. Data represent four Axin2 +/− mice and two Axin2 +/+ control mice. Error bars represent mean ±S.D. *P<0.05; **P<0.01; ***P<0.001.*

To determine the Wnt signaling activity, we performed the same experiment using Axin-2^{LacZ} heterozygous reporter cells, a well-established Wnt reporter mouse model.[36] Phenotypic analysis of the co-cultured cells exhibited similar T-cell development potential at different dosages of Wnt3a (data not shown). Importantly, the actual Wnt signaling activity of total Thy1+ cells correlated with the increasing concentration of Wnt3a (Figure 4b). Therefore, our data suggest that Wnt3a canonical Wnt signaling functions in a dosage-dependent fashion, in accordance with the differential Wnt signaling activity of the cells.

Wnt3a overexpression enhances B lymphopoiesis and Wnt5a overexpression augments myelopoiesis in vivo

To study the role of Wnt3a and Wnt5a signaling *in vivo* we carried out reconstitution assays in which we sorted CD45.2 LSK cells from fetal liver, transduced them with lentiviral vectors encoding Wnt3a, Wnt5a in combination with GFP, and transplanted them into irradiated CD45.1 B6-recipient mice (Figure 5a). At week 16 post transplantation we analyzed spleen, thymus and BM of the recipients. In the spleens of Wnt3a-overexpressing mice the percent-

age of B220+ CD19+ B cells was around twofold higher compared with the control mice within the GFP+ transduced compartment. In contrast, Wnt5a-overexpressing mice had around twofold higher percentage of CD11b+ Gr1+ myeloid cells relative to the control. Therefore, the ratio of B cells *versus* myeloid cells was in favor of B cells in the Wnt3a group, and in favor of myeloid cells in the Wnt5a group (Figures 5b and c).

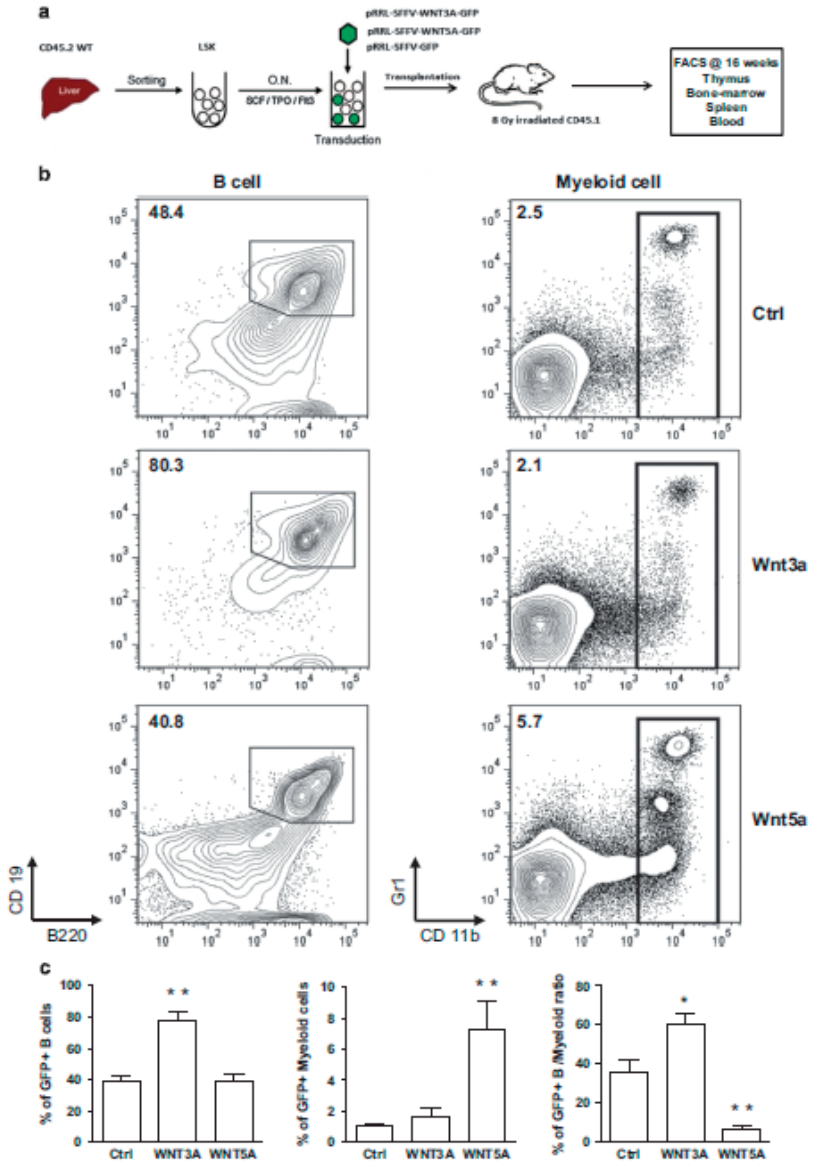


Figure 5. Wnt3a overexpression enhances lymphopoiesis, whereas Wnt5a overexpression augments myelopoiesis in vivo.

Figure 5. Wnt3a overexpression enhances lymphopoiesis, whereas Wnt5a overexpression augments myelopoiesis in vivo. *(continued)*

*(a) Experimental design. Lin– Sca1+ Kit+ (LSK) cells were sorted from E14 WT FL and were stimulated overnight in medium with SCF, TPO and Flt3-L cytokines. Next day, the cells were transduced with pRRL-SFFV-GFP (Ctrl), pRRL-SFFV-Wnt3a-GFP (Wnt3a) or pRRL-SFFV-Wnt5a-GFP (Wnt5a). Bulk of transduced cells were transplanted intravenously into CD45.1 8 Gy irradiated recipients. At week 16 post transplantation, the mice were killed and blood, spleen, BM and thymus were harvested and were assessed by FACS. Each group consists of five mice. (b) Representative FACS plots of B cell (B220+ CD19+) and myeloid cells (CD11b+ G1+) in the spleen of a recipient mouse 16 weeks after transplantation. The cells are pre-gated on CD45.2+ CD45.1− and GFP+. The numbers indicate percentage of cells within the gate. (c) Collective data represent the percentage of GFP+ B cells (left graph), myeloid cells (middle graph), and the ratio of B cells versus myeloid cells in the spleen of each group. Data are mean ±S.D. of five mice per group. *P<0.05; **P<0.01; ***P<0.001*

In the BM, the ratio of B cells *versus* myeloid cells was around threefold lower in the Wnt5a group, whereas there was no statistically significant difference between the control and Wnt3a group. MPPs (LSK Flt3+) were around threefold higher in the Wnt5a group compared with the control (Figure 6a). In the thymus, T-cell development was clearly blocked at early stages in the transduced compartment of the Wnt3a group, and to some extend with Wnt5a (as characterized by higher percentage and MFI of GFP in DN and ISP stages). However, the blocks did not affect the absolute number of mature T cells or total thymic cellularity (Figures 6b and c). Thus, Wnt3a overexpression induces B lymphopoiesis in spleen and BM whereas Wnt5a overexpression induces increased myelopoiesis in these organs.

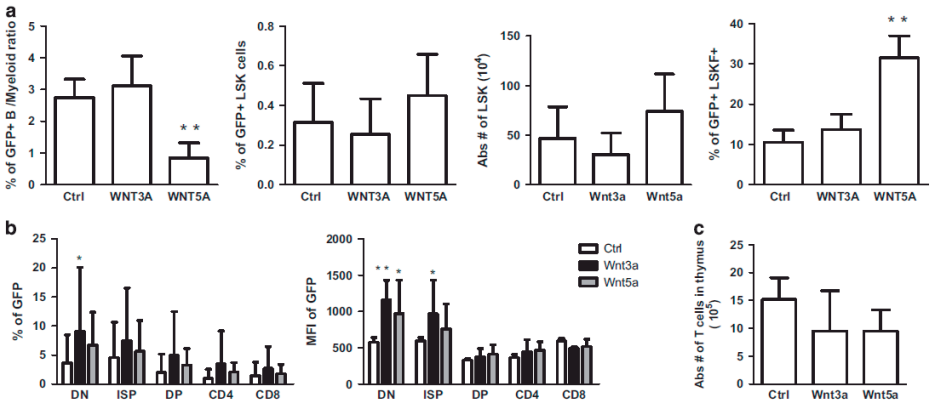


Figure 6. Wnt3a overexpression blocks T-cell development at early stages in the thymus.

*(a) At week 16 post transplantation, BM and Thymus are harvested and assessed by FACS Collective data depict the ratio of GFP+ B cells versus myeloid cells (Left), percentage and absolute number of LSKs (Middle), and percentage of LSK Flt3+[46] in the BM. Data are mean ±S.D. of five mice per group (right). (b) Collective data show percentage (left), or mean fluorescent intensity (MFI) (right) of GFP within each T-cell developmental stage for Ctrl group (White bar), Wnt3a group (Black bars) and Wnt5a group (gray bars). Data are mean ±S.D. of five mice per group. (c) Bar graph depict absolute number of total thymus (thymic cellularity) within each group. Data are mean ±S.D. of five mice per group. *P<0.05; **P<0.01; ***P<0.001*

Discussion

Canonical Wnt signaling has a well-established role in T-cell development in the thymus; yet only a few reports deal with non-canonical Wnt signaling. A side-by-side comparison of the effects of these interacting pathways has not been performed, in contrast to for instance B-cell development[20] and HSC biology.[37] We therefore set out to compare the prototypical canonical Wnt ligand Wnt3a with the prototypical non-canonical Wnt ligand Wnt5a. We demonstrated that the induction of canonical Wnt signaling via Wnt3a is first, important for T-cell development, and second functions in dosage-dependent fashion. Although intermediate-to-low doses of canonical Wnt signaling is beneficial for thymopoiesis, higher doses support B lymphopoiesis *in vivo*. On the other hand, Wnt5a non-canonical Wnt signaling induces myelopoiesis *in vivo* and it does not appear to function in a strict dosage-dependent fashion. Moreover, Wnt5a signaling induces apoptosis in developing thymocytes, in line with the diminished apoptosis observed in Wnt5a-deficient thymi.

Previous studies in our laboratory and many others revealed that canonical Wnt signaling is crucial for T-cell development, and it functions in a dosage-dependent fashion.[19] In the current study we used Wnt3a, a natural ligand of canonical Wnt signaling, and we obtained similar data. This shows that Wnt3a triggers canonical Wnt signaling via the *β*-catenin and TCF/LEF-dependent pathway in the thymus,[34] and that it is possible to modulate canonical Wnt signaling via differential concentration of Wnt3a physiologically. It is very likely that in the thymus, developing thymocytes are exposed to different types and concentrations of Wnt ligands. It is likely that thymocytes express different FZD receptors with various binding affinity to the existing Wnt proteins within the thymic microenvironment. Previous Q-PCR data suggest that this might be the case,[21] although experimental proof awaits the development of specific antibodies for each Frizzled receptor suitable for flow cytometry. As a result, developing thymocytes would undergo different levels of Wnt signaling owing to the accumulation of different amounts of *β*-catenin proteins in the cytoplasm.

Another possibility is that the interaction of canonical and non-canonical Wnt signaling might be important for control of *β*-catenin dosage in the cytoplasm. Sugimura *et al.*[37] elegantly described a situation where canonical and non-canonical Wnt signaling interact with each other in the hematopoietic system. Non-canonical Wnt maintains quiescent long-term HSCs through Flamingo and Frizzled 8 receptor on HSCs. Under stress, non-canonical Wnt is attenuated and canonical Wnt is enhanced, which results in the activation of HSC. The same might be true during T-cell development in the thymus under stress, which could be a fascinating issue for future studies.

Previously, Malhorta *et al.*[20] used a similar OP9-based Wnt3a/Wnt5 gain-of-function approach to study B lymphopoiesis. There are several differences between these two studies. (i) Their study is restricted to lympho-hematopoiesis of the BM and not thymopoiesis in the thymus.[34] Their study was confined to *in vitro* work using FACS-purified stem cells. Importantly, using a *β*-catenin overexpression approach, Kincade and coworkers[38] showed that committed B-cell progenitors gained myeloid lineage potential. Although we did not observe such phenomena in the T-cell lineage, Wnt3a clearly could maintain cells in an immature stage, at which thymocytes can still develop into alternative lineages. Thus, despite these differences, both studies suggest distinctive roles of Wnt family proteins during hematopoiesis and lymphopoiesis, and Wnt3a inhibits progenitor cell differentiation. However, the effect of Wnt5a on B-cell development is controversial. Malhorta *et al.*[39] showed that it induces B lymphopoiesis, whereas Liang *et al.* showed inhibition of B-cell proliferation. We did not observe any effect of Wnt5a on B-cell development even at higher doses (data not shown) which could be due to the difference in timing, source of stem cells or concentration of Wnt proteins.

Khoo *et al.*[40] have demonstrated that human aged HSCs have a reduced canonical Wnt signaling activity, and as a result impaired or delayed T-cell development, which is restricted to the T-cell progenitors, indicating the significance of Wnt signaling in human T-cell development during senescence. The notion that non-canonical Wnt signaling mimics murine HSC ageing has been put forward by Florian *et al.*[41]They reported that a shift from canonical to non-canonical Wnt signaling occurs during HSC ageing. Wnt5a treatment of young HSCs induced ageing associated stem cell apolarity, and an lymphoid to myeloid differentiation skewing, which is observed normally during ageing. We performed similar experiments in which we transduced HSC with Wnt3a and Wnt5a, rather than *ex vivo* treatment. Similarly, myelopoiesis was enhanced in Wnt5a-overexpressing HSC, and B lymphopoiesis in Wnt3a-overexpressing HSC (Figures 5 and and6).6). Therefore, Wnt3a treatment of stem cells before transplantation could be considered a promising approach to improve lymphopoiesis.

A series of studies in the laboratory of Perreault and coworkers[30, 31, 32] suggest that Wnt4 is a non-canonical Wnt ligand, enhances MPP expansion in the BM and ETP proliferation in the thymus, which results in increased thymic cellularity. We have also shown that Wnt5a overexpression induces MPP expansion, and DN expansion in thymus (Figure 6). However, this did not affect the total cellularity of the organs. This suggests specific roles of various Wnt family members during different stages of thymopoiesis, probably due to their differential expression throughout the thymus and/or differential responsiveness of developing thymocytes. For instance, Wnt4 has been proposed to function as a canonical Wnt in inducing FoxN1 expression in TEC,[42] but seems to act as a non-canonical Wnt in

studies aimed at improving thymic reconstitution from hematopoietic rather than thymic stromal cells.[30]

Previous studies indicate that deregulation of Wnt signaling occurs in leukemia.[43] We have also shown that deregulation of Wnt signaling due to the absence of Tcf1, induces lymphomas in mice.[44] Independent of our study, Yu *et al.*[11] also reported similar results and showed similarities of Tcf1 lymphomas with human ETP-ALL cases. In addition, Martins *et al.*[45] recently suggested that natural cell competition between young and old thymic progenitors is crucial for inhibition of T-ALL development. Therefore, thymic progenitor fitness is necessary for normal T-cell development. Our gain-of-function Wnt3a model preserves thymocytes in an immature state without inducing any malignancy. It is therefore intriguing to speculate that canonical Wnt signaling may be involved in regulating stemness of thymic stem cells.

Concluding, our work and that of others referred to above show discrete effects of Wnt3a and Wnt5a treatment on hematopoietic cells, both *in vitro* and *in vivo*. These attempts might lead to application of Wnt ligands as therapeutic candidates to improve HSC repopulation and T-cell reconstitution after SCT. However, the challenge and focus of future studies should be on determining the 'right concentration' of Wnt proteins to avoid deregulated Wnt signaling.

Materials and Methods

Lentiviral production and transduction

Wnt3a and Wnt5a gene transfer plasmids individually cloned into the multiple cloning sites of pRRL-SFFV-IRES-GFP lentiviral vector by restriction digestion and ligation reactions. 293 T cells were transiently transfected with either the genes transfer or empty control constructs together with helper plasmids using X-TremeGENE9 transfection reagent (Roche, Basel, Switzerland). Virus containing supernatants were harvested 20 h and 40 h after transfection in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, L-glutamin, 100 U/ml penicillin and 100 mg/ml streptomycin and used immediately for transduction, or stored at –80 °C for later use.

For transduction of OP9 stromal cells, 150 000 OP9-WT or OP9DL1 cells were seeded into a well of six-well plates with alpha MEM (Lonza, Verviers, Belgium) supplemented with 20% FCS-HI, L-glutamin and penicillin/streptomycin. The following day, OP9 cells were transduced with fresh or frozen viral supernatant containing 4 μg/ml proteamine sulfate (Sigma-Aldrich, St. Louis, MO, USA). After few days of culture transduced GFP+ cells were sorted, and stable OP9-pRRL-GFP (empty vector transduced), OP9-(DL1)- W3A (Wnt3a-transduced) and OP9-(DL1)-W5A (Wnt5a-transduced) cell lines were generated. Overexpression of the genes were then confirmed by Q-PCR gene analysis. The overexpression of DL1 gene was not altered in the transduced cell lines compared with untransduced or transduced with empty vector.

Coculture of fetal liver cells with OP9 cell lines

For different experiments various conditions of OP9 co-cultures were used as follows: OP9-WT, OP9-DL1-GFP, OP9-WT/DL1 (1 : 1), OP9-DLW3A, OP9-DLW5A, OP9-DLW3A/DL1 (1:1), OP9-DLW3A/DL1 (1 : 10) and OP9-DLW3A/DL1 (1 : 100). In all conditions 50 000 total fetal liver cells were cultured on confluent layers of OP9 cells, with AlphaMEM 10% FCS containing 50 ng/ml rmSCF, 10 ng/ml rmFlt3L and 10 ng/ml rmIL-7 (all cytokines from R&D systems, Minneapolis, MN, USA) in a well of 24-wells plate. For different purposes, cells were harvested after 6 h, 24 h, 3, 7 or 14 days of culture and stained for flow cytometric analysis.

In vivo transplantation assay

Transplantation assays were performed with the CD45.1/CD45.2 system. LSK cells were sorted from CD45.2 WT fetal liver mice, overnight stimulated in stemspan in presence of Flt3-L (50 ng/ml), TPO (10 ng/ml) and SCF (100 ng/ml). Next day, sorted LSKs were transduced by means of Retronectin (Takara Bio Inc., Kusatsu, Japan) with SFFV-IRES-GFP or SFFV-Wnt3a-IRES-GFP or SFFV-Wnt5a-IRES-GFP. The viral supernatants were titrated in advance to obtain equal transduction efficiency (~50%). One day after transduction 5×10^3 bulk transduced

LSK cells were transplanted intravenously into lethally irradiated (8 Gy) CD45.1 (9–12 weeks) mice together with 5×10^5 CD45.1 spleen support cells. Chimeras was analyzed at 4, 8 and 12 weeks after transplantation in peripheral blood, and mice were killed for analysis at 16 weeks post transplantation. Mice were considered repopulated when >1% multilineage CD45.2 cells could be detected in nucleated peripheral blood cells 3 months after transplantation.

Acknowledgments

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Supplemental Methods

Mice

Mice were bred and maintained in the animal facilities of Leiden University Medical Center, in accordance with legal regulations in The Netherlands and with the approval of the Dutch animal ethical committee. C57Bl/6-CD45.1 (Ly5.1) and C57Bl/6-CD45.2 (Ly5.2) mice were obtained from the Jackson Laboratory, and Conductin (Axin2)-LacZ mice were kindly provided by B. Jerchow and W. Birchmeier (Max Delbruck Center for Molecular Medicine, Berlin, Germany) and have been described previously [35].

Flow Cytometry

The following antibodies were obtained from BD Biosciences (San Diego, CA): anti-CD3-APC (145-2C11), anti-CD4-PeCy7 (RM4-5), anti-CD8-PerCP(53-6.7), anti-CD25-PE (PC61), anti-CD44-APC-Cy7 (IM7), anti-cKit-PeCy7 (2B8), anti CD11b-PE (M1/70), anti CD19-APC (ID3) . For Lineage depletion these markers were used: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), Gr1 (RB6-8C5), B220 (Ra3-6B2), Ter119 (Ly76) and Nk1.1 (PK136) biotin and subsequently were stained with streptavidin eFluor 450 (48-4317) from eBioscience. The following antibodies were also purchased from eBioscience: CD45.1-PE-Cy7 (A20), CD45.2 Alexa Fluor 780 (104), B220 PE-Cy7 (RA3-6B2), Thy1.2 APC (53-2.1), Gr1 eFluor 450 (RB6-8C5) and Sca1 PE-Cy7 (D7). Cells were stained in Fluorescenceactivated cell sorter (FACS) buffer (PBS, 2% bovine serum albumin, 0.1% sodium azide) for 30 min at 4 °C. Ultimately, Cells were washed and measured either on a Canto I, or an Aria (BD Biosciences). For apoptosis analysis cells were stained with 7AAD/AnnexinV (BD Bioscience). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular β-galactosidase activity was measured by staining cells with 2 mM fluorescein di-b-D-galactopyranoside (FDG) substrate (Molecular Probes). FDG was loaded into the cells by hypotonic shock at 37 °C for 1 min, prior to cell surface antibody staining. The β-galactosidase reaction was stopped with 1 mM phenylethyl b-D-thiogalactopyranoside (PETG, from Molecular Probes) after 3 hours as described before and optimized for use in thymocytes [36].

Gene expression analysis

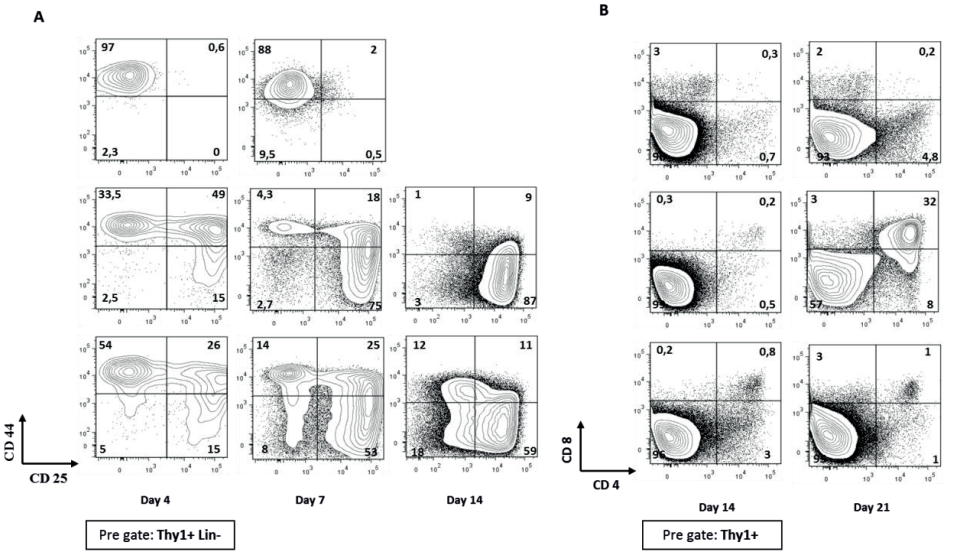
Total RNA was extracted using Qiagen RNeasy mini or micro columns. One ug of total RNA was used as a template for cDNA synthesis, using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), Oligo dT, and random hexamer primers. The RQPCR reaction was performed using TaqMan Universal mastermix (Applied biosystems, Foster City, CA, USA) and was run on a PRISM 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). The following primers were used in combination

with FAM-labelled probes from the universal probe library (Roche): Delta Like-1 forward primer: 5'- GGGGAGAGAGGGGAGAAGAT - 3'; reverse primer: 5'- ACAGCCTGGCAGACAAATG -3'; Wnt3a forward primer: 5'- CTTAGTGCTCTGCAGCCTGA -3'; reverse primer: 5'- GAGT-GCTCAGAGAGGAGTACTGG -3'; Wnt5a forward primer: 5'- ATGAAGCAGGCCGTAGGAC -3'; reverser primer: 5'- CTTCTCCTTGAGGGCATCG -3'; Axin2 forward primer: 5'-GCAG-GAGCCTCACCCTTC-3'; reverse primer: 5'- TGCCAGTTTCTTTGGCTCTT-3'. RQ-PCR results were normalized to Abl expression in the same sample: forward primer: 5'-TGGAGATAACACTC-TAAGCATAACTAAAGGT-3'; reverse primer: 5'-GATGTAGTTGCTTGGGACCCA-3'; and probe: 5'-FAM-CCATTTTTGGTTTGGGCTTCACACCATT- NFQ-3'.

Statistical analysis

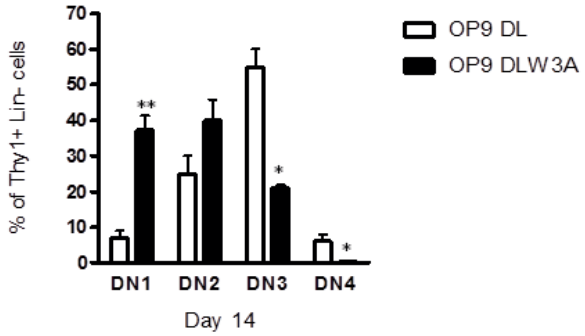
Statistical analysis was performed using the Mann–Whitney U test (Prism GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant. Asterisks indicate statistical significance as follows: * $P < 0.05$, and ** $P < 0.01$. Otherwise P values are mentioned.

Supplementary Figures



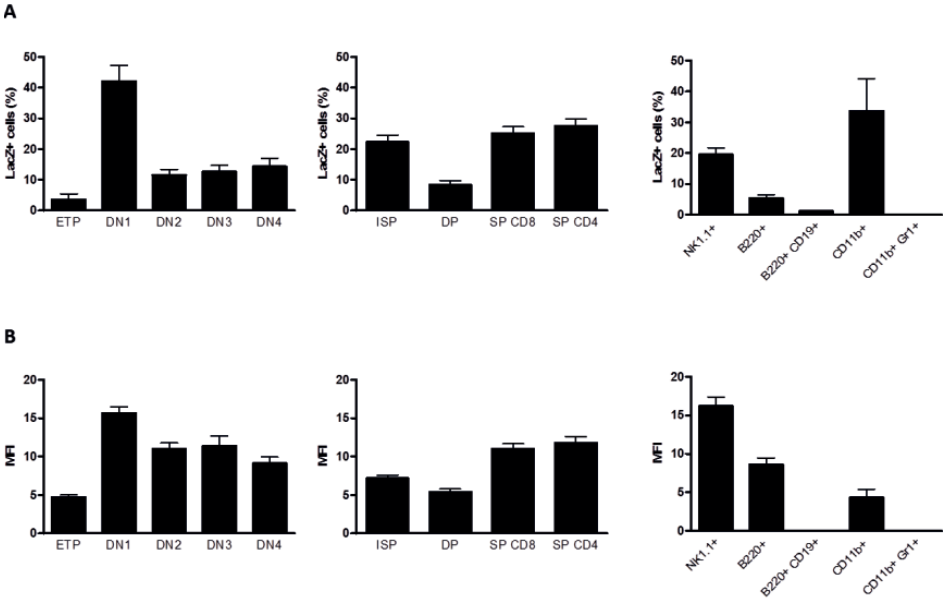
Supplementary Figure 1. Comparison of OP9WT/DL and OP9 Dl1 for T cell development support.

(A) *Total fetal liver cells were cocultured with OP9 WT (top row), OP9 DL1-GFP (middle row) or mix of OP9 WT and OP9 DL 1:1(bottom row). Cells were harvested after 4 days, 7 days and 14 days of coculture and were analysed by FACS for T cell development. The plots are pre-gated on Thy1+ LIN- markers and depict DN1-DN4 T cell developmental stages. The numbers represent percentage of each stage.* (B) *The cells were harvested 14 days and 21 days after coculture and were assessed by FACS for late stages of T cell development. The plots are pre-gated on Thy1+ marker. The numbers represent percentage of each population. Representative FACS plots of one independent experiment in triplicates are shown.*



Supplementary Figure 2. Canonical Wnt3a overexpression blocks T cell development at early stages.

Total fetal liver cells were cocultured with OP9 DL1 as control (white bar) or with OP9 DLW3A (black bars). Cells were harvested 14 days after coculture and were analysed by FACS for DN stages of T cell development. The percentage of DN stages are shown within Thy1+ LIN- populations. Error bars represent mean ± SD of two independent experiments, each in triplicates.



Supplementary Figure 3. In vivo measurement of canonical Wnt signaling activity in thymocytes and non-T cells within the Thymus.

Activation of the canonical Wnt signaling pathway was measured using the Axin2/conductinLacZ/+ Wnt-reporter mice by FACS. (A) Quantification of frequency, and (B) the mean fluorescence intensity (MFI) of the LacZ+ populations for each subset in the thymus are depicted. Littermate mice not carrying the reporter transgene (Axin2/Conductin+/+) were used to define the LacZ+ population. For each subset, MFI of the LacZ+ population was normalized for the MFI of corresponding LacZ- population (MFI LacZ+/MFI LacZ-) in order to correct for differences in background staining between different hematopoietic populations. Data represent results from three Axin2/conductinLacZ/+ mice and two Axin2/Conductin+/+ control mice, from one independent experiment. Error bars represent mean ± SD.

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CHAPTER 5

TCF1 REGULATES T LYMPHOCYTE LINEAGE FIDELITY THROUGH ITS TARGET GENES GATA3 AND BCL11B

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Abstract

T cell factor 1 (Tcf1) is the first T cell–specific protein induced in multipotent progenitors following Notch signaling in the thymus, leading to the activation of its two target genes, *Gata3* and *Bcl11b*. Tcf1 deficiency results in partial arrest in T cell development at various stages, high apoptosis, and increased development of B cells and myeloid cells. Phenotypically, fully T cell–committed Tcf1-deficient thymocytes have promiscuous gene expression and de-differentiate into immature thymocytes and non-T cells. Expressing Bcl11b in Tcf1-deficient cells rescues T cell development, but does not suppress the development of non-T cells; in contrast, expressing Gata3 suppresses the development of non-T cells, but does not rescue T cell development. These results reveal that T cell development is controlled by a minimal transcription factor network involving Notch signaling, Tcf1, and the subsequent division of labor between Bcl11b and Gata3, thereby ensuring a properly regulated T cell gene expression program.

Introduction

T cells are disease-fighting leukocytes that—similar to all blood cells—originate from hematopoietic stem cells (HSCs). However, whereas all other blood cell lineages develop in the bone marrow in specific niches , T cells develop in the thymus, a specialized organ located in the chest where progenitor cells migrate from the bone marrow and definitively commit to the T cell lineage, ultimately forming mature T cells [1]. The development of T cells within the thymus is a highly complex process involving successive stages in which the expression of CD4 and CD8 co-receptors occurs in distinct microenvironments [2]. Via a series of progressive developmental stages, T cell precursors (i.e., thymocytes) differentiate from double-negative (DN; $CD4^-CD8^-$) cells into intermediate immature single-positive (ISP; $CD8^+CD3^-CD4^-$) cells, then into double-positive (DP; $CD4^+CD8^+$) cells, and finally into single-positive (SP; $CD8^+CD4^-CD3^+$ or $CD4^+CD8^-CD3^+$) cells. In the DN stage, developing thymocytes can be further subdivided into four stages of differentiation based on their expression levels of CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Early stages are not committed to the T cell lineage (i.e., fate restricted), allowing alternate lineages to develop [3]. Indeed, B cells, dendritic cells, myeloid cells, and natural killer (NK) cells can all be generated from $CD44^+CD25^-$c-kit^{hi} early thymic progenitors (ETPs) [4,5], DN1 cells, and—albeit to a lesser extent—DN2 cells [6]. These multipotent cells, which can enter a number of differentiation programs, are directed towards the T cell lineage via a process called specification. The irreversible capacity to develop solely into T cells occurs somewhat later and is referred to as T lineage commitment; this process also involves the active repression of non-T cell lineages [7-9].

The microenvironment of the thymus provides a cellular context that drives T cell development. This process is initially driven by the expression of Notch ligands, particularly delta-like protein 4 (DLL4) [10], and later in the DP stage by providing the signals required to control positive selection (for self-MHC) and negative selection (against autoreactive T cell clones). The various stages in T cell development have been investigated in great detail using flow cytometry and genomic analyses; thus, T cell development serves as a paradigm for the molecular regulation of cell fate [11,12]. The fact that T cell development occurs in an anatomically separate niche has allowed researchers to study the detailed successive steps that underlie lineage specification and commitment. All of the events that establish the identity of T cell precursors are driven by Notch signalling [13], involving binding of the transcription factor RBP-J (also known as CBF1) to intracellular Notch ligands, thereby forming an active transcription factor complex in ETPs.

The subsequent stages in T cell development are governed by several key transcription factors that form an intricate gene regulatory network [14]. The core set of transcription

factors in the early phases of T cell development are Tcf1 (encoded by the gene confusingly termed *Tcf7*), Gata3, Bcl11b, and two members of the E2A family, Ikaros and Runx1 [14-17]. Importantly, the *Tcf7* gene is direct Notch signaling target and the first T cell–specific transcription factor induced by Notch signaling [18]; in contrast, Bcl11b drives T cell commitment by limiting the NK cell fate and activating the T cell developmental gene program at the DN2-DN3 stage [19], leading to expression of the fully rearranged TCR-beta gene in the DN3 stage. Rothenberg and colleagues showed that four transcription factors—Tcf1, Gata3, Notch/RBP-J, and to a lesser extent Runx1—are required for the timed expression of Bcl11b [14]. Of these four transcription factors, Tcf1 is the most complex, as it can act as both a transcriptional repressor (e.g., when bound by a co-repressor such as Groucho)or a transcriptional activator by binding β-catenin in order to respond to canonical Wnt signals [20]. Interestingly, Tcf1 also acts as a tumor-suppressor gene [21,22], and it can be functionally replaced—at least partially—by Lef1, a related transcriptional regulator expressed at approximately 50-fold lower levels than Tcf1 [23].

The precise role that Tcf1 plays in regulating T cell specification and commitment, and its interaction with other core regulatory factors in T cell development, is poorly understood. Therefore, we examined the role of Tcf1 in the earliest stages of T cell development, focusing primarily on fully committed DN3 cells. We found that Tcf1 is necessary for driving thymocytes down the T cell developmental path even after the T cell commitment stage, as Tcf1-deficient DN3 thymocytes can de-differentiate into DN1-like cells that can then develop into the myeloid and B cell lineages. In addition, we found that Tcf1 supports this "lineage fidelity" via two direct—and functionally complementary—target genes, *Gata3* and *Bcl11b*. An epistasis analysis using retroviral gene complementation in Tcf1-deficient stem cells revealed that the role of Gata3 in immature T cells is to repress B cell and myeloid fate, whereas Bcl11b establishes the T cell lineage program, and its expression can fully overcome the defect in T cell development inTcf1 deficient thymocytes.

Results

Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells

Tcf1 deficiency results in multiple incomplete blocks in T cell development that vary from mouse to mouse (Fig 1A). While the block at the ISP stage is well described[24-26], the earlier blocks have not been well documented. We therefore set out to better characterize these blocks and noticed developmental arrests at DN1, DN2, DN3 and ISP stages. (Fig. 1A). Although the block at the ISP stage has been well described [24-26], arrests at the earlier stages have not been reported in detail.. In addition to the partial blocks in development shown in Fig. 1A, we also observed increased percentages of non-T cell lineages, most notably B cells and myeloid cells (Fig. 1B). In contrast, Tcf1 deficiency did not affect the development of NK cells (Fig. 1C), consistent with previous reports that the development of NK cells is independent of the effect of Tcf1 on T cell lineage [27,28]. Consistent with T cell developmental arrest, loss of Tcf1 significantly impaired αβ T cell development (Fig. 1C).

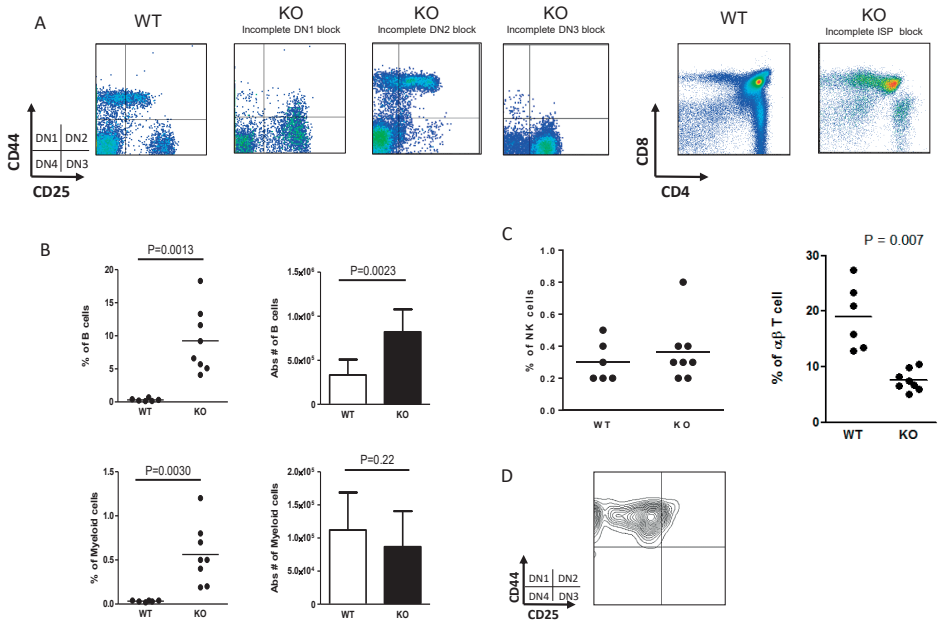

Figure 1. ***Tcf1 deficiency results in several incomplete blocks in T cell development and increased numbers of thymic B and myeloid cells***

A: Tcf1 deficient thymi can show different developmental arrests: DN1, DN2, DN3 or ISP. B: increased percentages of B cells and myeloid cells in Tcf1 -/- thymi. C: decreased numbers of αβT cells in Tc f1-/- thymi. D: Complete block at the DN1 to DN2 transition in the thymus of bone marrow chimeras generated from Tcf1 deficient stem cells.

In contrast to these partial arrests in developing mice, transplanting Tcf1-deficient stem cells into adult recipient mice led to a complete block in T cell development at the DN1-

DN2 transition (Fig. 1D), presumably of an insufficient compensatory expression of Lef1 in these cells [29]. Moreover, we cultured Tcf1-deficient stem cells on OP9-DL1 cells and found a similar DN1-like arrest, with increased numbers and relative percentages of non-T cell lineages (data not shown, but see Figs. 5 and 6).

Tcf1 deficiency leads to high levels of apoptosis

Developmental arrest in the thymus is often accompanied by an increase in apoptosis. To examine the role of apoptosis in Tcf1-deficient thymocytes, we measured apoptosis and proliferation of various developmental stages in the thymus of Tcf1-deficient mice and wild-type littermates. Compared to wild-type cells, we found increased levels of apoptosis in Tcf1-deficient cells at nearly every stage (Fig. 2), as well as decreased cell proliferation in the DN2 and DN4 stages (not shown).Taken together, these results explain the decrease in T cells in Tcf1-deficient mice; however, they do not explain the increased numbers of non-T cells.

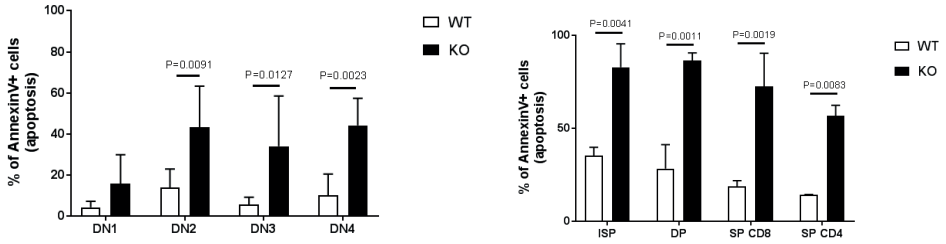

Figure 2: ***Increased apoptosis in Tcf1-/- thymocytes.***

Ex vivo Thymocytes were analysed by flow cytometry for various developmental subsets in combination with AnnexinV/7AAD. (n=4 or 5 per group)

Phenotypically, fully committed DN3 TCF1-deficient thymocytes have promiscuous gene expression and more open chromatin

Next, to better understand the role of Tcf1 in T cell commitment, we compared gene expression profiles between Tcf1-deficient thymocytes and wild-type thymocytes. The T cell commitment process starts at the DN2 stage and continues to the DN3a ($CD25^{+}CD44^{-}CD27^{-}$) stage in which a rearranged Tcrb gene is expressed in combination with pTA to form the pre-TCR complex in a process known as β-selection. After β-selection, the cells rapidly proliferate, express CD27, and are fully T cell committed based on expression of a functional, rearranged Tcrb gene [30]. We therefore performed whole-transcriptome RNA-Seq analysis on DN3b cells obtained from Tcf1-deficient and wild-type littermates (Fig 3A). For visualization the top 50 differentially expressed genes are shown and confirm absence of expression for Tcf7, but also that there were many fewer rearranged Tcrb genes than in wildtype control DN3b thymocytes, as indicated for the Trbj expressed gene segments(Fig. 3A). We used the genes differentially expressed

between Tcf1 deficient and wild type DN3b cells in a Gene Set Enrichment analysis (GSEA). We used published gene sets of T cell developmental stages to establish a DN2 signature [31]. The genes highly expressed in Tcf1-/- DN3b clustered strongly with DN2 cells, indicating that they share many characteristics of earlier developmental stages that are less T cell committed (Fig 3A). The RNA-seq data also indicated that many of the T cell commitment genes were low or not expressed while genes involved in for instance non T cell lineages (Pax5, Pu.1, Blc11a) were highly expressed. Based on these data we validated the expression of a number of important T cell developmental genes by Q-PCR on sorted DN1, DN2, DN3 and DN4 thymocytes. These results validated the RNA-Seq data and showed much lower expression of the T cell specific transcription factors Gata3 and Bcl11b (with higher expression of its functional counterpart Bcl11a) while the B cell commitment factor Pax5 and the myeloid associated factor Pu.1 were significantly higher expressed in the Tcf1 deficient thymocytes (Fig 3B). In addition, genes more associated with stem/progenitor cells (sometimes referred to as legacy genes[1]) such as c-kit were also significantly higher expressed(Fig 3B), while both Wnt and Notch target genes were decreased. Collectively, these data showed that while in some regards Tcf1-/- DN3 thymocytes were T cell committed, they also showed lineage infidelity, with expression of master regulatory genes from non-T cells. This notion was further substantiated by investigating the chromatin status of Tcf1 vs wildtype thymocytes using ATAC-Seq (Assay for Transposase-Accessible Chromatin sequencing) as discussed below.

Gata3 and Bcl11b are direct targets of Tcf1 and downregulated in Tcf1 deficient thymocytes

The downregulated mRNA expression levels of the transcription factors Gata3 and Bcl11b in various DN thymocyte stages in Tcf1 deficient mice, suggested that these factors may be direct target genes of Tcf1. In accordance, the Bcl11b and Gata3 enhancers contain conserved Tcf/Lef binding sites (see M&M). To check whether in DN thymocytes these promoters are regulated in a Tcf-dependent manner, we performed chromatin immune precipitation (ChIP) using a monoclonal antibody specific for Tcf1 (Fig 4A) followed by Q-PCR. This revealed binding of Tcf1 to the Gata3 and Bcl11b promoter sequences in wild type DN thymocytes but not in Tcf1 deficient thymocytes, showing that both genes are direct target genes of Tcf1. This finding was further substantiated by ATAC-Seq (Assay for Transposase-Accessible Chromatin) data which indicates chromatin accessibility Although in general, chromatin was less condensed in DN3b thymocytes lacking Tcf1 compared to wildtype DN3b cells, in certain specific areas the chromatin was more condensed. Focusing on the Bcl11b and Gata3 promoter/enhancer sequences, the chromatin in these promoters was less accessible compared to wild type littermate control DN3b cells (Fig 4B). Similarly, the TCRB loci were much less accessible in accordance with the RNA-Seq data. Interestingly, no major differences in chromatin accessibility were found at genes involved in alternative lineages, indicating that expression of these genes was not regulated at the level of chromatin opening.

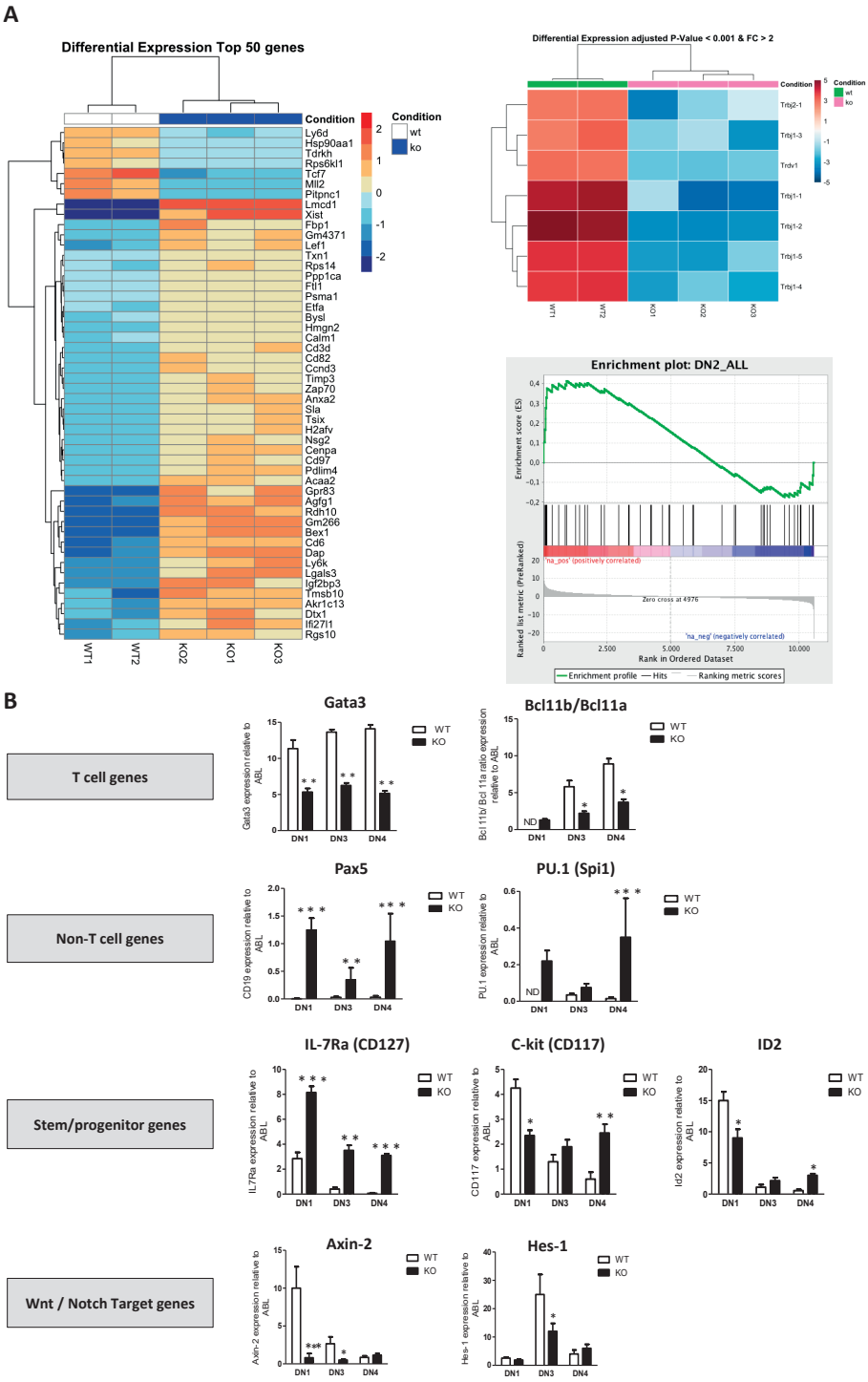
A
Differential Expression Top 50 genes
Condition
wt
ko
Ly6d
Hsp90aa1
Tdrkh
Rps6kl1
Tcf7
Mll2
Pitpnc1
Lmcd1
Xist
Fbp1
Gm4371
Lef1
Txn1
Rps14
Ppp1ca
Ftl1
Psma1
Etfa
Bysl
Hmgn2
Calm1
Cd3d
Cd82
Ccnd3
Timp3
Zap70
Anxa2
Sla
Tsix
H2afv
Nsg2
Cenpa
Cd97
Pdlim4
Acaa2
Gpr83
Agfg1
Rdh10
Gm266
Bex1
Cd6
Dap
Ly6k
Lgals3
Igf2bp3
Tmsb10
Akr1c13
Dtx1
Ifi27l1
Rgs10
WT1
WT2
KO2
KO1
KO3
Differential Expression adjusted P-Value < 0.001 & FC > 2
Trbj2-1
Trbj1-3
Trdv1
Trbj1-1
Trbj1-2
Trbj1-5
Trbj1-4
Enrichment plot: DN2_ALL
Enrichment score (ES)
'na_pos' (positively correlated)
Ranked list metric (PreRanked)
Zero cross at 4976
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
B
T cell genes
Non-T cell genes
Stem/progenitor genes
Wnt / Notch Target genes
Gata3
Gata3 expression relative to ABL
Bcl11b/Bcl11a
Bcl11b/ Bcl 11a ratio expression relative to ABL
Pax5
CD19 expression relative to ABL
PU.1 (Spi1)
PU.1 expression relative to ABL
IL-7Ra (CD127)
IL7Ra expression relative to ABL
C-kit (CD117)
CD117 expression relative to ABL
ID2
Id2 expression relative to ABL
Axin-2
Axin-2 expression relative to ABL
Hes-1
Hes-1 expression relative to ABL
WT
KO
DN1
DN3
DN4
ND

Figure 3: *Tcf1 deficient DN3b cells show promiscuous gene expression and more open chromatin loci compared to wild type littermate controls (continued)*

A: Heat map of the top 50 differentially expressed gene as determined by RNA-SEq of sorted DN3b cells from wild type and Tcf1 deficient thymi, with an extra focus on rearranged TCR products on the right. GSEA of the differentially expressed genes (Tcf1-/- over wild type for DN3b) are enriched for DN2 genes. B: Q-PCR validation of RNA-Seq data for selected T cell specific genes, genes expressed in non-T cells and legacy genes whose expression is inherited from stem cells/ multipotent progenitors

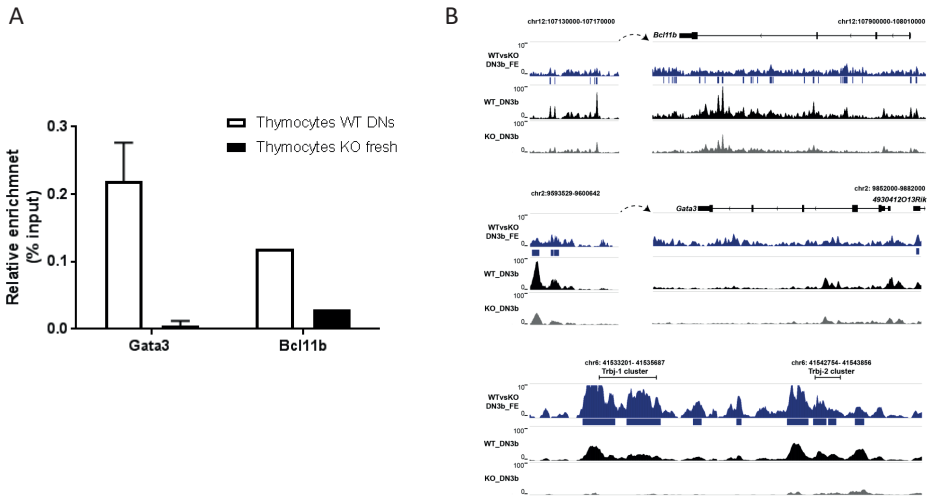

Figure 4: *Chromatin analysis in Tcf1 deficient vs. wild type DN thymocytes.*

A. Chromatin immune precipitation with an antibody specific for Tcf1 revealed that the Gata3 and Bcl11b promoters are occupied by Tcf1 in vivo, whereas in Tcf1-/- DN thymocytes no binding can be detected. Negative controls with IgG instead of anti-Tcf1 showed no enrichment. B. ATAC-Seq data mined for the Bcl11b, Gata3 and TRB-J genomic regions. Per locus the relative abundance of transposase accessible regions is indicated. In blue the fold enrichment(FE) of wildtype over Tcf1-/- DN3b cells are indicated, blue bars underneath show the significant peaks called by MACS2. In black the individual ATAC-seq profile from each genotype is shown. Data are shown as normalized read density.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes de-differentiate into DN1 thymocytes, B cells, and myeloid cells

Based on the hypothesis derived from these results, that Tcf1 deficient DN3 thymocytes may not be T cell committed, we sought to better investigate the differentiation capacity of Tcf1-/- DN3 thymocytes. Therefore, DN3 cells were sorted and cultured under conditions with strong T cell inducing capacity. Indeed the majority of wild type DN3 thymocytes differentiated further into DN4 cells, with a smaller part remaining DN3 (Fig 5A). Unexpectedly, most Tcf1-/- DN3 thymocytes dedifferentiated into DN1 and DN2 cells, with extensive B and myeloid development while only a minority of cells remained DN3 without any further

development along the T cell lineage (Fig 5A). Especially development into B cells was extensive, with up to 60% of DN3 thymocytes developing into B cells (Fig 5B).These DN1 and DN2 cells were not a contaminating fraction that grew out, as intracellular staining for Tcrb revealed high Tcrb expression in these DN1/2 cells at similar levels as wild type DN3 and DN4 cells (Fig 5B). We conclude that Tcf1KO cells dedifferentiate to less committed cells and exhibit lineage infidelity with significant development into alternative (non-T) lineages. When ETP cells rather than DN3 cells were seeded on OP9-DL1, as expected Tcf1 deficient cells were arrested in development at DN1, with abundant B and myeloid development, whereas wild type stem cells differentiated along the T cell lineage with many fewer non-T cells (Suppl Fig 1)

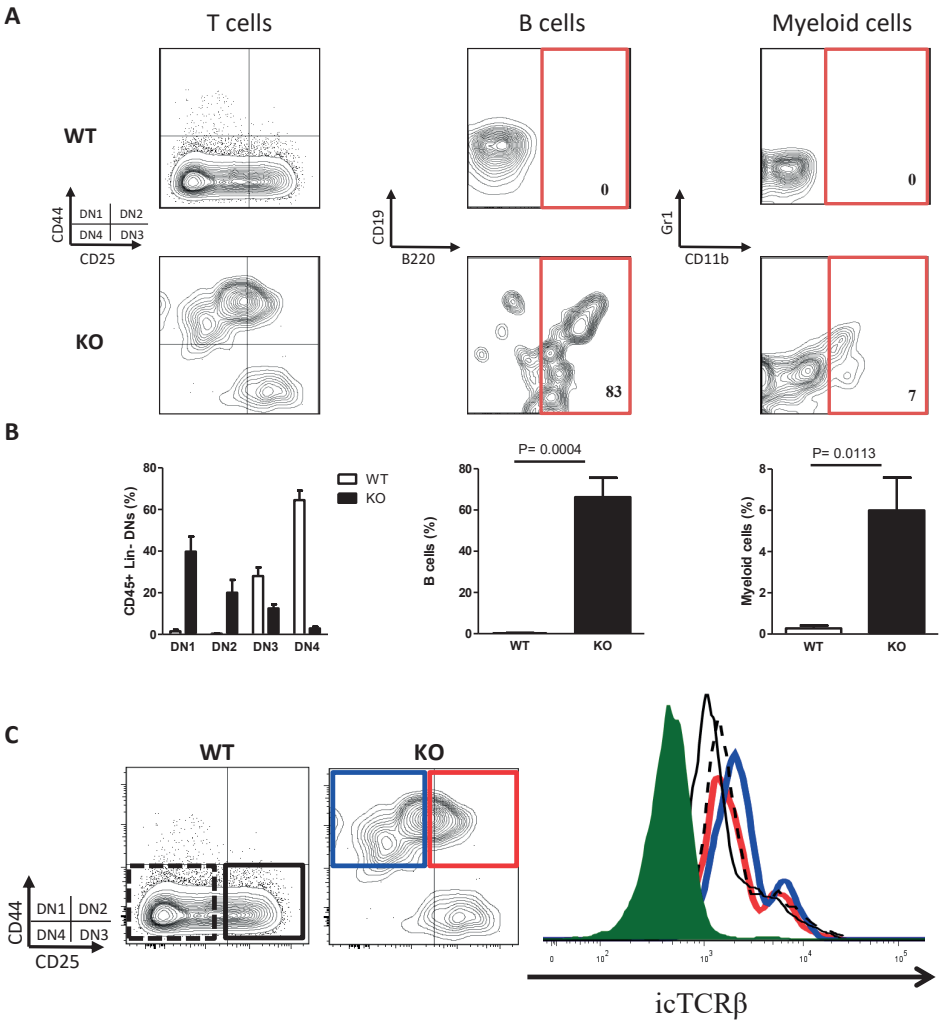

Figure 5: ***Tcf1 deficient DN3 cells de-differentiate into DN1/2 cells with multipotent lineage capacity (continued)***

A: wild type DN3 cells sorted and seeded on OP9-Dl1 cells develop largely further into DN4 or remain DN3, while Tcf1 deficient cells develop into DN1 and DN2 cells with prominent B cell and myeloid cell development B: Quantification of the developmental plasticity nad de-differentaion effcts of DN3 Tcf1 deficient thymocytes into DN1, DN2, myeloid and B cells. C: Intracellular TCRβ staining reveals the dedifferentiated DN1 and DN2 cells to be derived from DN3 cells

De-differentiation into alternate lineages can be prevented by expressing Gata3 in Tcf1 deficient thymocytes

Epistasis analysis is a powerful genetic tool, often used in model organisms such as Drosophila to investigate hierarchical relationships between genes[32]. It can be more complex to perform in mammals such as mice, where not only expression per se but also gene dosage is important. For instance, while complete loss of Gata3 blocks T cell development at the earliest stages, transgenic overexpression of Gata3 can lead to development of mast cells in the thymus[33-36]. We therefore expressed Gata3 and Blc11b using recombinant retroviruses as they have a broad range of expression that would allow different phenotypes to be selected under the strong developmental pressure of the thymic microenvironment. We used retroviruses encoding GFP and Gata3 or Bcl11b together with GFP to investigate complementation of the Tcf1 phenotype by either Gata3 or Bcl11b(Fig 6A,7A). We used retroviruses solely encoding GFP as negative controls.

Expression of Gata3 could partially rescue the development of Tcf1-/- thymocytes from a DN1 arrest to DN2 but not further(Fig 6B). Strikingly, Gata3 strongly suppressed the enhanced development of B and myeloid cells (granulocytes as well as monocytes) from Tcf1-/- as well as the less prominent non T development from wild type thymocytes (Fig 6C). The suppression of B cell development was also observed in vivo when Gata3 completed Tcf1-deficient stem cells were transplanted in irradiated recipient mice (Fig 6D, right and supplemental Fig 2A). However, thymic T cell development again was arrested at a DN1/2 transition, barely different than GFP control transduced cells (Fig 6D, left; supplemental fig 2B). Thus, the major role of Gata3 in earliest DN development is the suppression of non T cell development with only a minor feed forward role into the T cell program.

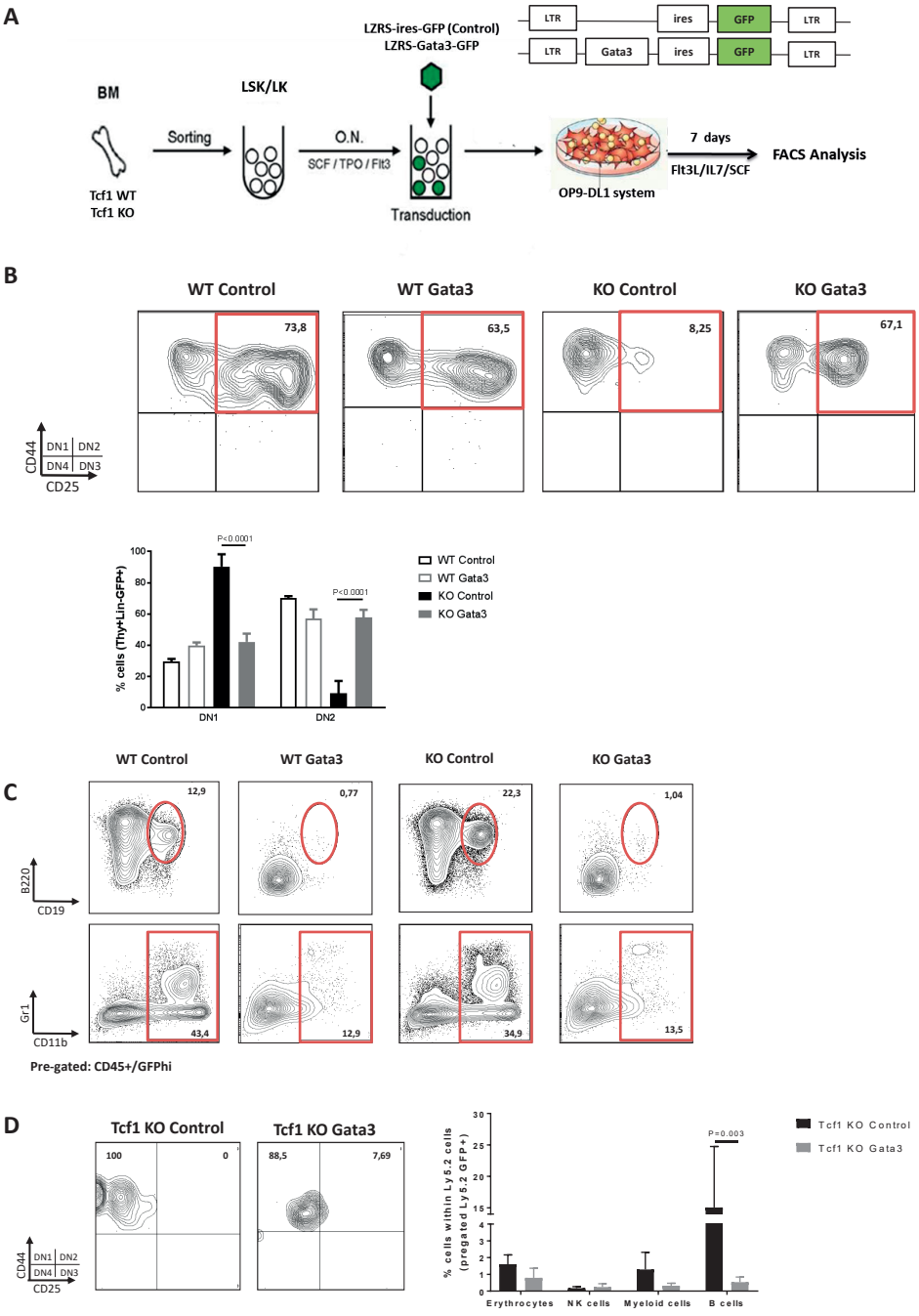
A
LZRS-ires-GFP (Control)
LZRS-Gata3-GFP
LTR
ires
GFP
LTR
LTR
Gata3
ires
GFP
LTR
BM
LSK/LK
Sorting
O.N.
SCF / TPO / Flt3
Transduction
7 days
Flt3L/IL7/SCF
FACS Analysis
OP9-DL1 system
Tcf1 WT
Tcf1 KO
B
WT Control
WT Gata3
KO Control
KO Gata3
73,8
63,5
8,25
67,1
CD44
CD25
DN1
DN2
DN4
DN3
% cells (Thy+Lin-GFP+)
P<0.0001
P<0.0001
DN1
DN2
WT Control
WT Gata3
KO Control
KO Gata3
C
WT Control
WT Gata3
KO Control
KO Gata3
12,9
0,77
22,3
1,04
B220
CD19
Gr1
CD11b
43,4
12,9
34,9
13,5
Pre-gated: CD45+/GFPhi
D
Tcf1 KO Control
Tcf1 KO Gata3
100
0
88,5
7,69
CD44
CD25
DN1
DN2
DN4
DN3
% cells within Ly5.2 cells (pregated Ly5.2 GFP+)
P=0.003
Erythrocytes
NK cells
Myeloid cells
B cells
Tcf1 KO Control
Tcf1 KO Gata3

Figure 6: ***Re-expression of Gata3 suppresses B and myeloid development in Tcf1 deficiency. (Continued)***

A:layout of retroviral complementation experiments with GFP and/or Gata3

B: Gata3 expression partially overcomes the DN1 thymocyte block and suppressed the enhanced non-T cell lineages after 7 days in the OP9-Dl1 culture system (Two-way ANOVA) Error bars represent the SD from two independent experiments. C: in vivo complementation reveals suppression of B cell development also in the spleen, but minimal and partial rescue of T cell development in the thymus (D)(two-way ANOVA) Error bars represent the SD from 3-4 individual mice per group.

The T cell lineage-specific defects caused Tcf1 deficiency can be rescued by expressing Bcl11b

Bcl11b(Fig 7A) in contrast rescued the T cell development of Tcf1 deficient cells virtually completely. Bcl11b transduced Tcf1 deficient stem cells developed readily into Thy1 positive (Fig 7B) cells, and could develop into DN2 and DN3 thymocytes to a similar degree as wild type thymocytes (Fig 7C). However, overexpression of Bcl11b did not influence B and myeloid development from Tcf1 deficient cells.

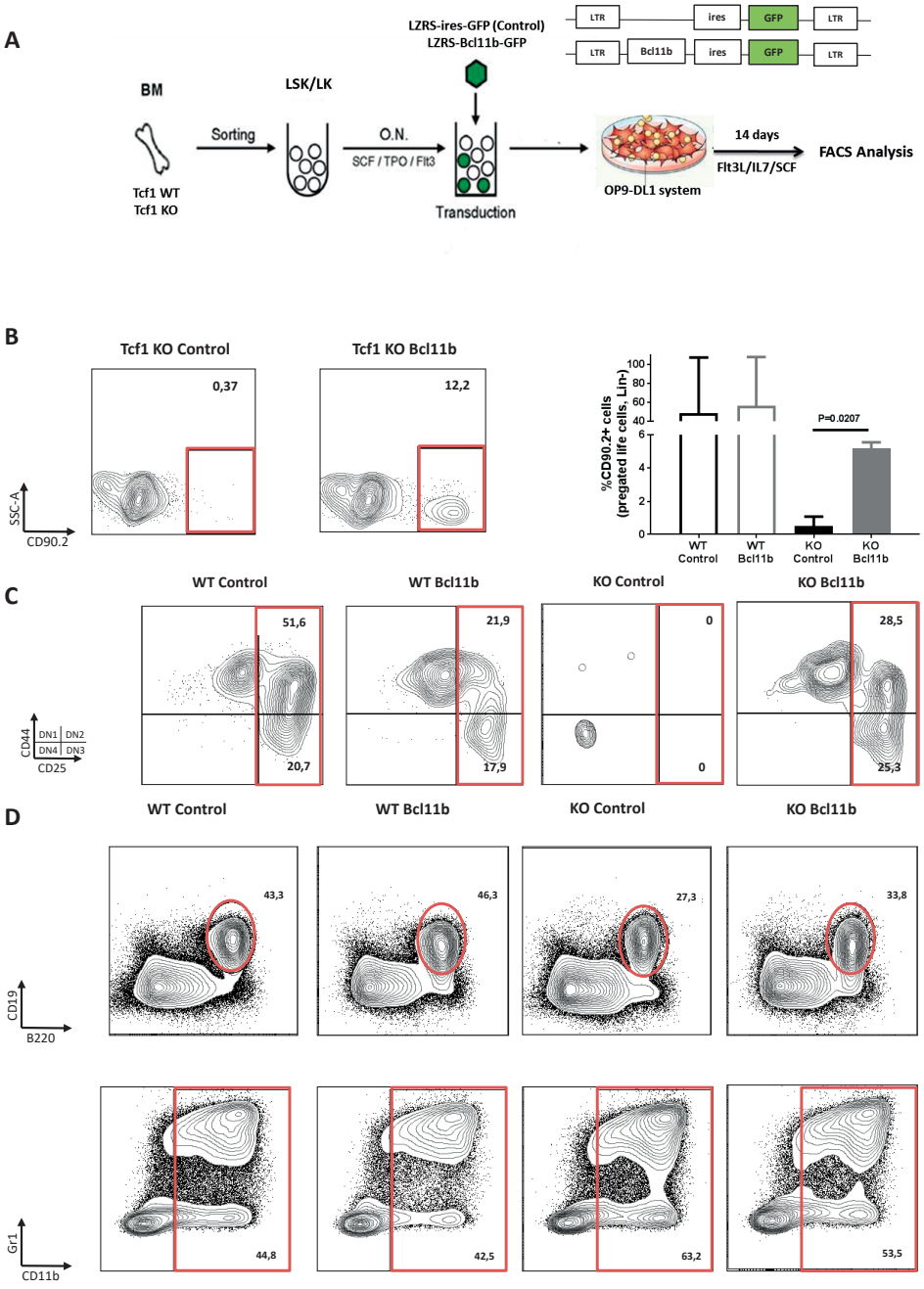

Figure 7: ***Re-expression of Bcl11b rescues T cell development from Tcf1 deficient stem cells.***

A: layout of retroviral complementation experiments with Bcl11b. B: Thy1 expression is rescued in Tcf1 deficiency by expression of Bcl11b after 14 days in OP9-Dl1 culture. C: Bcl11b fully rescues T cell development from Tcf1 -/- stem cells that otherwise are arrested in DN1. D: Bcl11b overexpression does not affect myeloid and B cell development. (Two-way ANOVA) Error bars represent the SD from three wells in two independent experiments.

Discussion

T cell development has been used as a classic example of a relatively ordered pathway to study cell fate determination [16], thereby giving the impression that transcriptional regulation during T cell development is a well-understood process. Despite this general belief, however, and compared to other developmental processes (for example, B cell development, which has similar requirements in terms of proliferation, lineage restriction, immune receptor rearrangement, and checkpoints for premature and mature immune receptors), the roles of the major transcription factors in T cell development are rather poorly understood. In B cell development, a clearly defined linear hierarchical relationship exists between E2A, EBF1, and Pax5 [37-44]. However, with respect to early T cell development, whether the Notch (RBP-J), Gata3, Bcl11b, Runx1, E2A, Tcf1/Lef1, Ikaros, and/or Hox genes play unique, redundant, or synergistic roles remains unclear and is the subject of intense research that focuses largely on either individual factors or the collective activity of these factors using computational biology. Considering that Notch signaling is required for T cell development, and given that the first T cell–specific target gene is *Tcf7* [18], which encodes Tcf1, we investigated the process of T cell lineage commitment in Tcf1-deficient mice.

Although Tcf1-deficient mice have been studied extensively, a comprehensive description of developmental arrest in these mice is currently lacking. Here, we report that developing thymocytes in adult Tcf1-deficient mice have incomplete arrest at several stages, including the previously reported ISP block [26].

The study of Tcf1-deficient mice is generally complicated by three factors. First, in the absence of Tcf1, the HMG box transcription factor Lef1—which is expressed in the thymus, albeit at much lower levels than Tcf1—plays a compensatory role [23,29,45]. This low-level expression of Lef1 causes incomplete penetrance of the Tcf1-deficient phenotype. However, if Tcf1-deficient stem cells are either transplanted into recipient mice or cultured on OP9-DL1 cells to induce T cell differentiation, complete block occurs at the DN1 stage (see Fig. 1D). Therefore, in our experiments we used bone marrow-derived cells obtained from Tcf1-deficient mice. Second, Tcf1-deficient mice are prone to developing T cell lymphomas in the thymus [22], which is similar to T-cell acute lymphoblastic leukemia (T-ALL) in patients. As discussed above, this issue can be overcome by using Tcf1-deficient stem cells instead of thymocytes. The third issue associated with studying Tcf1-deficient mice is that Tcf1 functions as both a transcriptional repressor and a transcriptional activator (for example, when bound to the Wnt mediator β-catenin). Indeed, when Tcf1-dependent promoters were tested using *in vitro* reporter systems, transcription occurred only when β-catenin was also expressed [46,47]. Consistent with this notion, Tcf1 binds to the promoter/enhancer regions of the target genes *Gata3* and *Blcl11b*, and we found evidence that Tcf1 binds to

β-catenin at these promoter regions (data not shown). In addition, DN stages of T cell development show high canonical Wnt signaling, which is driven by β-catenin and Tcf/Lef [48]. On the other hand, some of Tcf1's functions in the earliest stages of T cell development are independent of β-catenin [18], possibly due to the redundant role of Lef1.

A seminal study by Busslinger and colleagues revealed that Pax5 is a major lineage commitment factor in the development of B lymphocytes [42,43,49]. Thus, B cells that lack Pax5 can de-differentiate into multipotent progenitor cells that can replenish all hematopoietic lineages, even *in vivo*. In this respect, our findings are somewhat analogous, as Tcf1-deficient DN3 cells—which should be fully committed—have promiscuous gene expression and can de-differentiate into immature cells that can give rise to non-T cell lineages, including B cells and myeloid cells. Indeed, key transcription factors that drive alternate lineages (e.g., the transcription factors Bcl11a, Pax5, and Pu.1) are robustly expressed in Tcf1-deficient DN3 and DN4 cells, but not in wild-type cells. In contrast to wild-type cells—in which global chromatin accessibility is restricted in order to ensure that only T cell lineage genes are expressed—Tcf1-deficient cells have many more accessible loci, thereby explaining their functional role as multipotent progenitor cells. In contrast with Pax5-deficient cells, however, only a small number of Tcf1-deficient cells survive the de-differentiation process, which is likely due to the high level of apoptosis in Tcf1-deficient thymocytes.

Given that both *Bcl11b* and *Gata3* are key target genes for Tcf1, we expressed these transcription factors in Tcf1-deficient cells in an attempt to rescue the thymic phenotype. Similar analyses of epistasis have been used previously in model organisms (e.g., *Drosophila*) to delineate both hierarchical and functional relationships. The expression of exogenous *Gata3* has been shown to suppress B cell development in the wild-type thymus [50]; however, we found that Gata3 also suppresses myeloid fate in DN thymocytes. Interestingly, Gata3 does not suppress myeloid fate in the bone marrow, even though the effect on B cell development occurs outside of the thymus.

Our finding that the constitutive expression of Bcl11b in Tcf1-deficient cells fully rescued T cell development suggests a division of labor between Bcl11b and Gata3, with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T cell–specific genes. Taken together, the data from our group and others indicate a gene network in which Notch signaling via RBP-Jk drives the expression of Tcf1, which in turn activates Gata3 and Bcl11b, most likely in collaboration with Notch signals that can also act directly on these genes' promoters. Interestingly, in addition to its requirement for initiating the T cell commitment process, Tcf1 expression is also required to maintain lineage fidelity. In skin stem cells, lineage infidelity increases the likelihood of malignancy [51]. Thus, given that loss of Tcf1 leads to the rapid development of T cell lymphomas, lineage infidelity may also serve as a previously unrecognized factor in leukemogenesis.

Materials and Methods

Mice

C57Bl/6 TCF-1 -/- ΔVII/ΔVII mice were originally described by Verbeek et al (1995) and C57Bl/6-Ly5.1 mice were purchased form Charles Rivers Laboratories. Mice were bred and maintained in the animal facility of Leiden University Medical Center. All animal experiments were performed in accordance with legal regulations in The Netherlands and with approved protocols of the Dutch animal ethical committee.

Mice used for transplantation assay were kept in specified pathogen-free section and were fed with special food and antibiotic water. Genotyping assay of newborn Tcf1 mice was performed with DNA samples from earpieces using GoTaq Flexi DNA polymerase kit (Promega) according to manufacturer's instructions.

Flow cytometry and cell sorting

Single cell suspensions from thymus, spleen, BM and blood were stained with monoclonal antibodies against CD3e, CD4, Cd8a/Ly-2, CD11b/Mac-1, CD19, CD25, CD27, , CD44/Ly-24, CD45.1/Ly-5.1, CD45.2/Ly-5.2, B220/CD45R, CD90.2/Thy1.2, CD117/c-kit, CD135/Flt3, Gr1/Ly-6G-6C, NK1.1, Sca1/Ly-6A, TCRβ, and Ter-119/Ly-76 (See STAR Table_ Antibodiesfor additional antibody information). All antibodies used were directly conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7 or efluor450. Biotinylated antibodies were revealed with streptavidin conjugated antibodies (PE, efluor450, APC-Cy7, APC or Pe-Cy7) (all antibodies were purchased from BD or eBioscience).

Cells were blocked with normal mouse serum (NMS, Invitrogen) for 10min at room temperature and subsequently cell surface staining was performed in two steps. Firstly, cells were incubated for 30min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30min incubation step at 4°C was performed with the streptavidin conjugated antibodies mix.

Cell apoptosis was assessed by AnnexinV and 7AAD staining, which was performed following the PE AnnexinV Apoptosis detection Kit protocol (BD Pharmingen) after the cell surface staining. Proliferation assay was done by intracellular Ki67 staining (mIgG as control) with PE Mouse anti-human Set protocol (BD Pharmingen). For that purpose, cells were initially stained for cell surface markers as described previously and subsequently fixated and permeabilized by using fixation/permeabilization buffer (eBiosience) for an

hour at 4°C. Cells were then washed with permeabilization (eBiosience) buffer with 2% NMS and stained with Ki67 or IgG1 solution for 30min at 4°C in the dark.

Double positive CD4&CD8 cells before DN cell sorting and lineage positive cells before LSK/LK sorting were depleted using magnetic-activated cell sorting, autoMACS (Miltenyi Biotec). For DNs sorting, thymocytes were first stained with anti-CD4 and CD8-biotin, following by Streptavidin microbeads staining according to manufacturer instruction (Miltenyi Biotec). For LSK/LK cell sorting, lineage depletion kit (Miltenyi Biotec) was used according to manufacturer instruction. Subsequently, depleted cells were stained again for DNs or LSKs as described before. Cell sorting was performed on FACSAria II (BD Biosciences) or stained cells were measured with FACS-CantoII (BD Bioscience). Data was analysed using FlowJO (Tree Star). All different hematopoietic populations were defined as described in Table S2_Appendix.

Cell culture

Bone-marrow-derived stromal cell line OP9 and OP9-DL1 cells which ectopically express the Notch ligand Delta-Like 1 (DL1) were used as described by J.C. Zuñiga-Pflucker. Sorted DN cells were cultured on OP9 or OP9 WT/OP9-DL1 (10:1) confluent monolayers in αMEM (Lonza)-10%FCS, 1% P/S (Life Technologies) and GlutaMAX (Life Technologies) medium complemented with 50 ng/ml rmFlt3L, 50 ng/ml rmSCF, 10 ng/ml rmIL-7, and 50μM β-mercaptoethanol (β-ME; Sigma-Aldrich). (all cytokines purchased from R&D). Cells were harvested after 7 to 14 days of coculture and were analysed by flow cytometry.

Transduced LSK and LK with LZRS-ires-eGFP (control), LZRS-Gata3-eGFP or LZRS-Bcl11b-GFP vector were cultured on OP9-DL1 monolayer for 6 to 14 days in αMEM-10%FCS complemented with rmIL7 (10 ng/ml),rm Flt3L (50 ng/ml), rmSCF (10 ng/ml) and β-ME (50μM). Harvested cells were analysed by flow cytometry or sorted.

Retroviral production

LZRS-Gata3 and Bcl11b plasmids were obtained from Addgene and cloned into LZRS-ires-eGFP vector (Addgene, control vector). Control, Gata3 and Bcl11b retroviruses were generated using Phoenix ecotropic packaging cell line (ATTC). Cells were cultured in IMDM (Lonza)-10%FCS-1% Penicillin/Streptomycin -1%Glutamine and transfected with plasmids using X-treme Gene9 DNA transfection reagent (Roche) protocols. Selection of transfected cells was performed with 1mg/mL puromycin (Sigma-Aldrich) for a week and viral supernatant was harvested at 24h and 48h.

Retroviral transduction

LSK and LK sorted cells were stimulated overnight in StemSpan serum-free expansion medium (StemCell Technologies) supplemented with 10ng/ml rmTPO (R&D) , 50ng/ml rmFlt3L (R&D) and 100ng/ml rmSCF (R&D). Antibiotic mix with polymixine B sulphate, kanamycin, penicilin/streptomycin and amphotericin B was added to prevent bacterial infections. Hematopoietic progenitors were transduced using RetroNectin (Takara Bio Inc) coated wells according to the manufacturer's instructions. Non-tissue culture plates were coated with RetroNectin overnight at 4ºC and then blocked with 2% bovine serum albumin (BSA) in PBS for 30min. Retroviral supernatant (24h or 48h) was centrifuged at 1500xg for 2h at 32ºC and incubated an extra hour at 37ºC. After coating, viral supernatant was removed and stimulated cells were immediately added on the virus-coated plates. Cells were cultured in StemSpan medium supplemented with rmTPO (10 ng/ml), rmFlt3L (50 ng/ml) and rmSCF (100 ng/ml) and transduced overnight at 37ºC. LZRS-ires-eGFP,LZRS-Gata3-ires-eGFP and LZRS-Bcl11b-ires-eGFP transduced cells were used for *in vitro* and *in vivo* approaches.

Quantitative real time (rt)-PCR

RNA from sorted cells was purified using Micro RNeasy kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). RT-PCR was performed using TaqMan Universal Master Mix II in combination with specific probes for indicated genes from Universal Probe Library (Roche). Specific primers for ABL-2, Bcl11a, Bcl11b, Gata3, Pax5, PU.1/Spfi1, IL-7Ra, CD117/c-kit, ID2, Axin-2 and Hes1. were designed and purchased from Sigma-Aldrich (See specific gene sequences on Table 3_Appendix). Samples were analyzed by StepOnePlus RT-PCR system (Life Technologies). Relative transcript abundance was determined by ΔCt and expression levels were normalized for the endogenous reference gene ABL-1. All samples were run in at least in duplicates.

RNA-SEQ

RNA from sorted DN3b cells ($Lin^-CD25^+CD44^-CD27^+$) from Tcf1-/- and wild type littermates thymi was isolated using the Mini RNeasy Kit (Qiagen) The integrity (scores > 9.0) of the RNA was determined on the Agilent 2100 Bioanalyzer (Agilent). Total RNA enrichment for sequencing poly(A) RNAs was performed with the TruSeq mRNA sample preparation kit (Illumina). 1µg of total RNA for each sample was used for poly(A) RNA selection using magnetic beads coated with poly-dT, followed by thermal fragmentation. The fragmented poly(A) RNA enriched samples were subjected to cDNA synthesis using Illumina TruSeq preparation kit. cDNA was synthesized by reverse transcriptase (Super-Script II) using poly-dT and random hexamer primers. The cDNA fragments were then blunt-ended through an end-repair reaction, followed by dA-tailing. Subsequently, specific double-stranded bar-coded adapters were ligated and library amplification for 15 cycles was performed. The pooled cDNA library

consisted of equal concentration bar-coded samples. The pooled library was sequenced in one lane, 36 bp single read on the HiSeq2500 (Illumina).

RNaseq data were aligned to the mm10 genome using the STAR aligner (Dobin et al., 2013) and quantified using featureCounts . The raw counts data were processed using the "voom" function (Law et al., 2014) in the limma R package which normalizes the data and assigns a weight for each measurement for subsequent linear model fitting.. Differential expression was assessed using the limma moderated T statistic.. Normalization for replicate number and technical parameters was also applied directly to the voom result to obtain "normalized counts," which were used for data visualization. Geneset enrichment was performed using the "RankSumWithCorrelation" function in the limma R package, which automatically corrects enrichment statistic inflation due to correlation among genes

ATAC-SEQ

15,000 sorted DN3b Cells were washed 1 time with cold PBS. Pellets were spun down at 500 g for 5 min at 4°C, and the supernatant was removed carefully. 20 µl of transposase mix (10µl 2xTD buffer, 1 µl TDE (Nextera DNA Library Prep Kit; Illumina), 0.2 µ digitonin (G9441, Promega), 8.8 µl nuclease-free water) was added to the cells. Reactions were incubated at 37°C for 30 min. Transposed DNA was purified using the MinElute Reaction Cleanup Kit (28204, Qiagen), amplified, and again purified according to published protocols (Buenrostro et al 2015, CurrProtocMolBiol). Size selection was done using Low Range Ultra Agarose (161-3107, Bio-Rad). Fragments between 150-600 bp in size were used for further analysis. Quality and quantity of the libraries was assessed by Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent) before sequencing. Libraries were sequenced 50 bp, paired-end, on a HiSeq4000.

Reads were mapped to mm10 using bwa mem(Li H et al 2010, Bioinformatics) with default settings. Only reads with high mapping quality (Q>10) were used for further analysis, and DNA duplicates were removed using samtools(Li H et al 2009, Bioinformatics). Data were aggregated by each unique genotype and subsampling was done to correct for sequencing depth(about 23million reads). Differential peaks(in bed file format) between WT and Tcf1 KO cells were called using MACS2 with the following parameters: -g mm -B –nomodel (q-value <0.05). BigWig-tracks were generated by MACS2 using WT as input and Tcf1 KO as control. The track of Fold Enrichment (WT over KO) was generated using the bdgcmp function in MACS2 with the following settings: -m FE.

Chromatin immunoprecipitation

DN thymocytes ($CD8^-CD4^-$) from Tcf1-/- and wildtype littermates were sorted and subsequently crosslinked with formaldehyde (Sigma). Crosslinking was quenched with Glycine and after cell lysis chromatin was sonicated into fragments. Sonicated chromatin was precleared

and incubated with antibodies. TCF-1 (C46C7; #2206 Cell Signalling Technologies). Immuno precipated chromatin complexes were purified and quantified by real-time PCR using Fast-start Universal Sybr Green Master mix (Roche)

Competitive transplantation assay

Competitive transplantation assay is used to determine HSC development and functionality in vivo by measuring multi-lineage reconstitution of hematopoiesis in irradiated transplanted mice. This assay was performed with the Ly5.1/Ly5.2 (CD45.1/CD45.2) system. 12.5000 transduced LSK and 40.000 transduced LK cells from Ly5.2 mice were transplanted into lethally irradiated (8.07Gy) Ly5.1 mice (8-12 weeks), together with 300.000 spleenocytes (Ly5.1) as support cells. Chimerism and peripheral T cell were analysed at week 6 after transplantation in peripheral blood. Mice were sacrificed for analysis 7 weeks after transplantation to evaluate hematopoietic system repopulation. Mice were considered repopulated when ≥1% multi-lineage Ly5.2 cells could be detected. Single cell suspension from the thymus, spleen and bone marrow (BM), as well as lysate blood were analysed by flow cytometry as described previously.

Statistical methods

All statistics were calculated and all graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by standard two-tailed Student t test t student (*$p<0.05$) or ANOVA.

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Author contributions

Conceptualization: FF, LGP, FJTS.; Methodology, FJTS, KPO, LCD.; Investigation: FF, LCP, MvE, HW,MB, MC, MMT.; Writing – Original Draft, FJTS.; Writing – Review & Editing, FF, LCP, MB, MC, MMT, KPO, LCD, FJTS Resources, MB, MC.; Supervision, FJTS, KPO, LGP. Authors have no conflict of interest to report.

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CHAPTER 6

The Nuclear Effector of Wnt-Signaling, Tcf1, Functions as a T-Cell–Specific Tumor Suppressor for Development of Lymphomas

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Abstract

The HMG-box factor Tcf1 is required during T-cell development in the thymus and mediates the nuclear response to Wnt signals. Tcf1$^{-/-}$ mice have previously been characterized and show developmental blocks at the CD4−CD8− double negative (DN) to CD4+CD8+ double positive transition. Due to the blocks in T-cell development, Tcf1$^{-/-}$ mice normally have a very small thymus. Unexpectedly, a large proportion of Tcf1$^{-/-}$ mice spontaneously develop thymic lymphomas with 50% of mice developing a thymic lymphoma/leukemia at the age of 16 wk. These lymphomas are clonal, highly metastatic, and paradoxically show high Wnt signaling when crossed with Wnt reporter mice and have high expression of Wnt target genes *Lef1* and *Axin2*. In wild-type thymocytes, Tcf1 is higher expressed than Lef1, with a predominance of Wnt inhibitory isoforms. Loss of Tcf1 as repressor of Lef1 leads to high Wnt activity and is the initiating event in lymphoma development, which is exacerbated by activating Notch1 mutations. Thus, Notch1 and loss of Tcf1 functionally act as collaborating oncogenic events. Tcf1 deficiency predisposes to the development of thymic lymphomas by ectopic up-regulation of Lef1 due to lack of Tcf1 repressive isoforms and frequently by cooperating activating mutations in *Notch1*. Tcf1 therefore functions as a T-cell–specific tumor suppressor gene, besides its established role as a Wnt responsive transcription factor. Thus, Tcf1 acts as a molecular switch between proliferative and repressive signals during T-lymphocyte development in the thymus.

Introduction

Cancers often develop as consequence of deregulated expression of key factors that operate during normal development. Deregulation of the Wnt signaling pathway has been implicated in many types of malignancies, especially in solid tumors (reviewed in [1]–[3]). Mutations in different components of the Wnt pathway are found to contribute to carcinogenesis [3]. During normal development, Wnt proteins function as proliferation-inducing growth factors and may also affect cell-fate decisions [4]–[6]. Wnt proteins bind to their Frizzled receptors, thereby preventing proteosomal degradation of the Wnt mediator β-catenin. Subsequently, β-catenin is translocated to the nucleus, where it forms an active transcription complex with the nuclear proteins downstream of the Wnt pathway: TCF1 (T-cell factor 1, the product of the Tcf7 gene, referred to as Tcf1 throughout this article), LEF1 (Lymphocyte-Enhancer-binding Factor), or the homologous factors TCF4 and TCF3. All TCF/LEF factors belong to a family of high-mobility-group (HMG) proteins that utilize the HMG box for sequence-specific DNA binding. The HMG boxes of these factors are virtually identical and likely display indistinguishable DNA-binding specificities [7],[8]. TCF/LEF nuclear proteins exist as transcriptional repressors and only upon binding to β-catenin will form an active transcription complex. For TCF1, at least eight isoforms have been identified with different capacities to bind β-catenin, thereby influencing the responsiveness of cells toward Wnt signals [9]. The long isoforms of TCF1 contain the amino-terminal β-catenin-binding domain, whereas the shorter isoforms lack this domain and will therefore function as the naturally occurring repressors of the pathway.

A large body of evidence has shown that canonical Wnt signaling is essential for thymocyte proliferation and normal T-cell development [10]–[16]. Among the Wnt proteins, specifically Wnt1 and Wnt4 are essential for thymocyte proliferation [11], which is reflected in mice deficient for Wnt1 and Wnt4 that display low thymic cellularity [15]. In addition, overexpression of Wnt4 selectively expands thymic output from transduced hematopoietic stem cells [17]. Recently, we showed that another Wnt protein, Wnt3a, plays a crucial role in fetal thymopoiesis, with Wnt3a$^{-/-}$ thymi showing severely reduced numbers of DP and a block of the preceding CD8+ Immature Single Positive (ISP) stage [18], thereby displaying an exact phenocopy of fetal thymi in Tcf1$^{-/-}$ mice.

Studies on mice deficient for the Wnt-responsive nuclear proteins reveal crucial roles for Tcf1 in T-cell development and Lef1 in B-cell development [19],[20]. Tcf1$^{-/-}$ mutant mice have a severe reduction of thymic cellularity and a partial block in thymocyte differentiation at the transition from the CD8+ ISP stage to the CD4+CD8+ double positive (DP) stage [19]. Thymocytes of Tcf1$^{-/-}$ mice do not proliferate as strong as their wild-type counterparts [21]. These data indicate that lack of Tcf1 mainly results in lack of prolifera-

tion and therefore expansion of the thymocytes. Although Lef1$^{-/-}$ mice have normal T-cell development, mice deficient in both Lef1 and Tcf1 have a complete block in T-cell differentiation at the ISP stage, which indicates redundancy between these two factors [22]. The block in T-cell development in Tcf1-deficient mice was shown to be caused by lack of Wnt mediated signals, as Tcf isoforms without the β-catenin binding domain could not restore T-cell development, but Tcf isoforms containing the interaction domain with the Wnt mediator β-catenin fully reconstituted T-cell development [23]. In addition, soluble Frizzled receptors acting as inhibitors of Wnt signaling [11], or overexpression of an inhibitor of the interaction between β-catenin and Tcf/Lef factors, ICAT [13], inhibited T-cell development at the same stages as Tcf1 KO mice. Recent studies by the laboratories of Bhandoola and Gounari further emphasize the importance of Tcf1 as a critical regulator of T-lineage specification and differentiation. These investigators demonstrate that Tcf1 is critical for induction of a T-cell-specific gene program in stem cells and uncommitted progenitors [16]. In addition, the Gounari lab showed that ETPs lacking Tcf1 fail to develop normally [10]. Together, these studies conclusively point to Tcf1 as an essential transcriptional regulator of T-cell specification, commitment, and lineage determination [24]. Here we report that Tcf1, besides acting as a Wnt responsive transcription factor, also has an important other function, namely as tumor suppressor for the development of T-cell lymphomas.

Results

Lack of Tcf1 Induces Thymic Lymphomas with High Frequency

The generation of mice lacking the Wnt-responsive factor Tcf1 revealed a crucial role for Tcf1 in T-cell development [19]. $Tcf1^{-/-}$ mice have thymi characterized by low cellularity (fewer than 10^7 cells at 6–8 wk of age, compared to $>10^8$ cells in littermates [19]), which is due to the blocks at the DN and ISP developmental stages. Strikingly, over time an increasing number of $Tcf1^{-/-}$ mice were found with an extremely enlarged thymus (example in Figure 1A). The occurrence of these enlarged thymi in $Tcf1^{-/-}$mice was not a rare finding. Studying the thymi of 150 $Tcf1^{-/-}$ mice showed a clear bimodal distribution in thymic cellularity (Figure 1B). A threshold in thymocyte numbers occurs at 18×10^6 thymocytes. A cellularity of $<18\times10^6$ cells can therefore be regarded as a normal size $Tcf1^{-/-}$ thymus, whilst a thymus with a cellularity $>18\times10^6$ cells can be regarded as an abnormal enlarged thymus. The right graph of Figure 1B demonstrates an increasing percentage of $Tcf1^{-/-}$ mice with a hyperplastic thymus with increasing age. This hyperproliferation could be caused by increased normal proliferation or by the presence of a clonal population of tumor cells. Results described below collectively demonstrate that these cells are neoplastic in nature and represent thymic lymphomas (Figures 1C and 2). Immunohistochemical analysis shows that neoplastic cells completely disrupted the thymic architecture (Figure 1C), and loss of corticomedullary demarcation was evident. Neoplastic cells invaded the thymic capsule, neighboring adipose tissue, thoracic organs, liver, kidney, spleen, and lymph nodes. Abdominal organs (liver and kidney) and lymphatic tissues (spleen and lymph nodes) have been shown to be preferential sites for metastasis of systemic lymphomas [25].

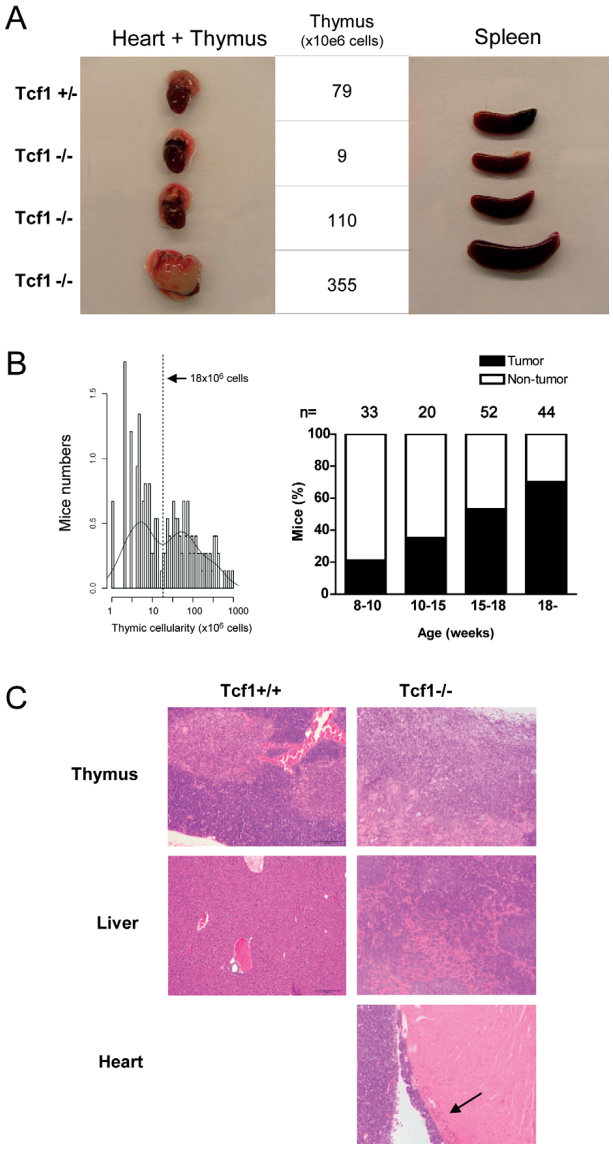

Figure 1. Tcf1$^{-/-}$ mice develop thymic lymphomas.

(A) Heart with thymus and spleen of one Tcf1$^{+/-}$ and three Tcf1$^{-/-}$ mice are shown. The number of thymocytes of the mice is from top to bottom: 79, 9, 110, and 355×10^6 cells. (B) Tcf1$^{-/-}$ mice are sacrificed at a certain age (8–10, 10–15, 15–18, or >18 wk), and according to the bimodal distribution of the thymus size, mice were considered to have a tumor when the thymus contains >18×10^6 cells. In total 150 Tcf1$^{-/-}$ mice were analyzed. The percentage of mice categorized to have a tumor is shown for each age group in the right panel. (C) Histopathology of normal tissue compared to tumors from Tcf1$^{-/-}$ mice. Paraffin sections of thymus, liver (Tcf1$^{+/+}$ and Tcf1$^{-/-}$), and heart (Tcf1$^{-/-}$ only) were stained with hematoxylin and eosin (HE). The HE sections of the Tcf1$^{-/-}$ mouse show neoplastic cells arranged in cords and sheets in the thymus that infiltrate in the liver and the heart; final magnification, 100×.

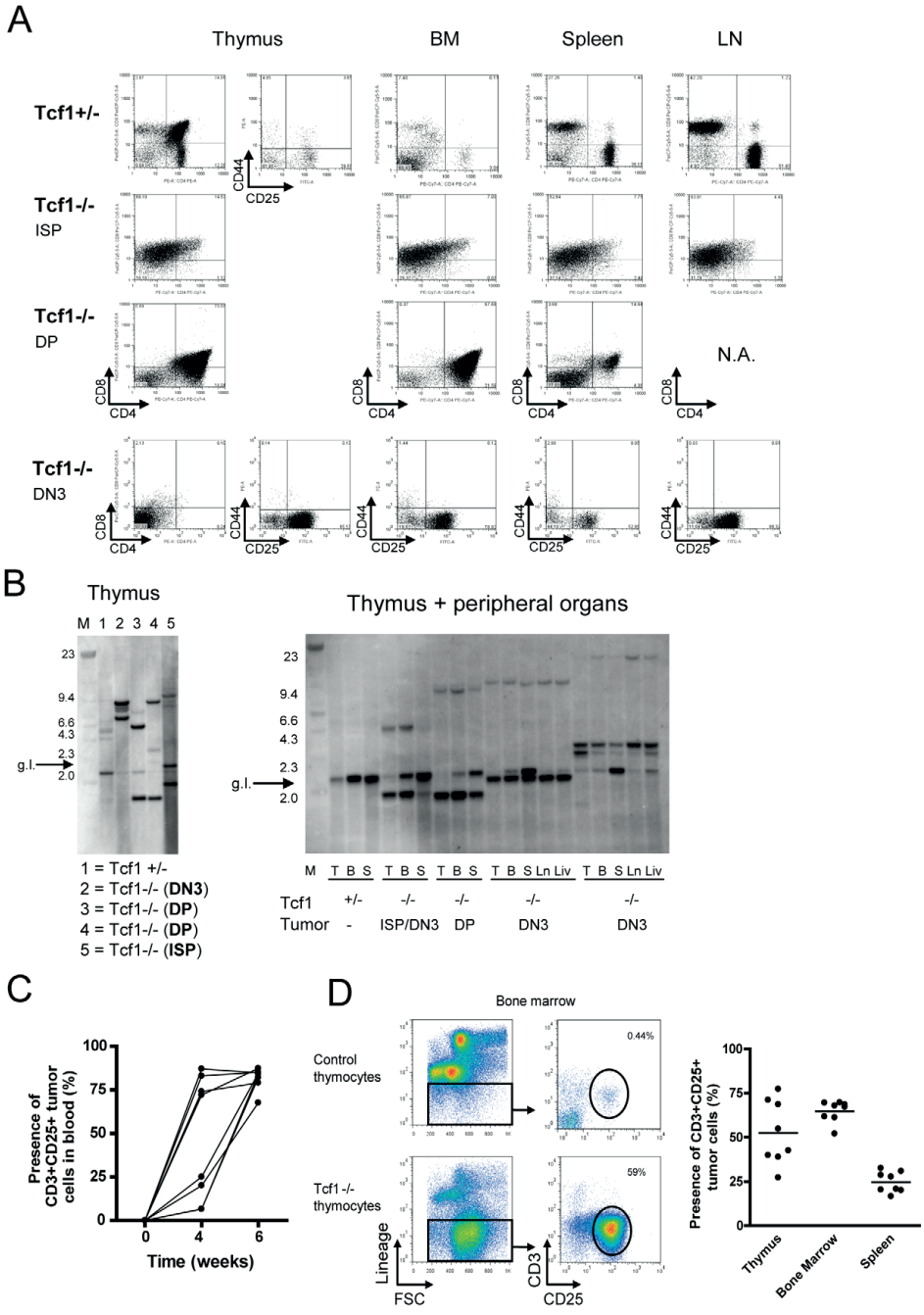

Figure 2. Tcf1$^{-/-}$ thymic lymphomas are phenotypically heterogeneous, malignant, and oligoclonal.

Figure 2. Tcf1$^{-/-}$ thymic lymphomas are phenotypically heterogeneous, malignant, and oligoclonal. (continued)

(A) Thymocytes, bone marrow (BM) cells, splenocytes, and lymphocytes obtained from lymph nodes (LN) were stained for CD4, CD8, CD44, and CD25. The CD4/CD8 and CD44/CD25 plot is shown for lineage negative cells for all the different organs. Results are shown for one Tcf1$^{+/-}$ mouse and three representative Tcf1$^{-/-}$ mice. (B) Left panel, genomic DNA was prepared from thymocytes derived from a Tcf1$^{+/-}$ mouse (Lane 1, showing the germline band and a faint pattern of additional bands) and four different Tcf1$^{-/-}$ mice with thymic lymphomas (DN3 tumor, Lane 2; DP tumors, Lanes 3 and 4) and an ISP tumor (Lane 5). The asterisk indicates the fragment expected for the germ-line (g.l.) TCRβ gene configuration. On the left side, a size marker (M) was included of which the sizes are indicated in the figure (kD). Right panel, genomic DNA was prepared from different organs, T (thymus), B (bone marrow), S (spleen), LN (lymph nodes), and Liv (liver) of one control Tcf1$^{+/-}$ mouse and four different Tcf1$^{-/-}$ mice. The phenotype of the lymphoma as determined by FACS analysis is shown for each mouse. The first lane includes a size marker (M) of which the sizes (kD) are indicated in the figure. (C) Tcf1$^{-/-}$ tumor cells characterized by an intermediate expression of CD3 and CD25 were injected into sublethally irradiated Rag1$^{-/-}$ mice by tail vein injection. Mice were bled at the time of injection, 4 and 6 wk after injection. The presence of CD3+CD25+ tumor cells in blood is indicated per mouse (n=8). (D) Six weeks after transfer of the cells, the mice were sacrificed and the thymus, bone marrow, and spleen were analyzed for the presence of tumor cells. Cell suspensions were stained for lineage markers (Mac1, Gr1, B220, Ter119, and NK1.1) and CD4, CD8, CD3, CD44, and CD25. An example of the gating strategy is shown for a bone marrow sample. The percentage of CD3+CD25+ tumor cells is shown per mouse per organ.

Tcf1−/− Induced Thymic Lymphomas Are Clonal and Highly Metastatic

The lymphomas have different phenotypic characteristics that to some extend reflect the developmental blocks. Thus, the different lymphomas in the Tcf1$^{-/-}$ thymi were categorized into several phenotypically distinct subgroups or mixtures thereof: DN1, DN3, ISP, or the DP stage (examples of DN3, ISP, and DP lymphomas are shown in Figure 2A). The different phenotypes are not correlated to the age of the mice, and their frequency is: 5% DN1, 32.5% DN3, 40% ISP, and 22.5% DP (n = 40 Tcf1$^{-/-}$ tumor mice). The thymocytes overpopulating the thymus were present in other hematopoietic organs such as spleen, bone marrow, and lymph nodes (Figure 2A), suggesting the high malignant capacity of these cells to invade other organs as expected from the size of the organs. To examine whether this aggressive proliferation of thymocytes was due to clonal expansion, a Southern Blot analysis was performed, using the Jβ2 region of the TCRβ gene. In contrast to DNA from a Tcf1$^{+/-}$ control thymus, which shows the germline band (g.l. indicated by the arrow) and a heterogeneous mix of bands characteristic of a polyclonal cell population (Figure 2B, left panel, Lane 1), the lymphoma samples only showed between two and four distinct bands (germline and one or more rearranged alleles), indicating that they consisted of one or two independent clones (Figure 2B, left panel, Lanes 2–5). Interestingly, the same clonal band was found in the metastases in the secondary organs, BM, spleen, liver, and lymph nodes (Figure 2B, right panel). To further confirm the malignancy of these thymic lymphomas and their ability to grow autonomously and invade the organs in secondary recipients, 5×10^5 thymocytes were

transferred into sublethally irradiated Rag1$^{-/-}$ recipients. The malignant donor thymocytes were derived from Tcf1$^{-/-}$ mice, characterized by intermediate expression of CD3 and CD25, and control donor thymocytes were obtained from Tcf1$^{+/-}$ mice. Four weeks after transfer, the tumor cells (as characterized by the expression of CD3 and CD25) were present in peripheral blood in 50% of the recipient mice (Figure 2C). Six weeks after transfer, all animals were sacrificed. Recipients receiving the malignant thymocytes of Tcf1$^{-/-}$ origin all displayed an enlarged liver and spleen, and tumor cells were detectable by flow cytometry in all organs tested (thymus, BM, spleen; Figure 2D). Together these results demonstrate that a lack of Tcf1 predisposes mice to a high risk of developing thymic lymphomas, which are clonal and characterized by an aggressive metastatic phenotype. These results indicate that Tcf1 functions as a tumor suppressor gene in the thymus.

Tcf1–/– Thymic Lymphoma Cells Exhibit Deregulated Wnt Pathway

To gain insight into the molecular mechanism underlying the Tcf1-deficient tumor development, we compared the gene expression profile of thymocytes derived from Tcf1$^{-/-}$ mice with tumors, Tcf1$^{-/-}$ mice of similar age without tumors, and control Tcf1$^{+/-}$ mice. Samples of 17 mice were studied by genome-wide expression profiling using Affymetrix microarrays, namely five control Tcf1$^{+/-}$ mice, four Tcf1$^{-/-}$ mice without tumor, and eight thymic tumors from Tcf1$^{-/-}$ mice. Expressions of several oncogenes, known to be involved in leukomogenesis, were analyzed and were not up-regulated in the thymic lymphomas compared to the Tcf1$^{-/-}$ without lymphomas (*Tal1*, *Tal2*, *Lyl1*, *Lmo1*, *Lmo2*, *SilTal*, *p53*; unpublished data). Analysis of components of the Wnt pathway confirmed that *Tcf1* (*Tcf7*) expression was absent (as expected) in the Tcf1$^{-/-}$ thymocytes (with and without a tumor), whilst in all but one Tcf1$^{-/-}$ tumor sample, the expression level of the transcription factor Lef1 was up-regulated compared to control (Tcf1$^{+/-}$) thymocytes (Figure 3A, left panel).

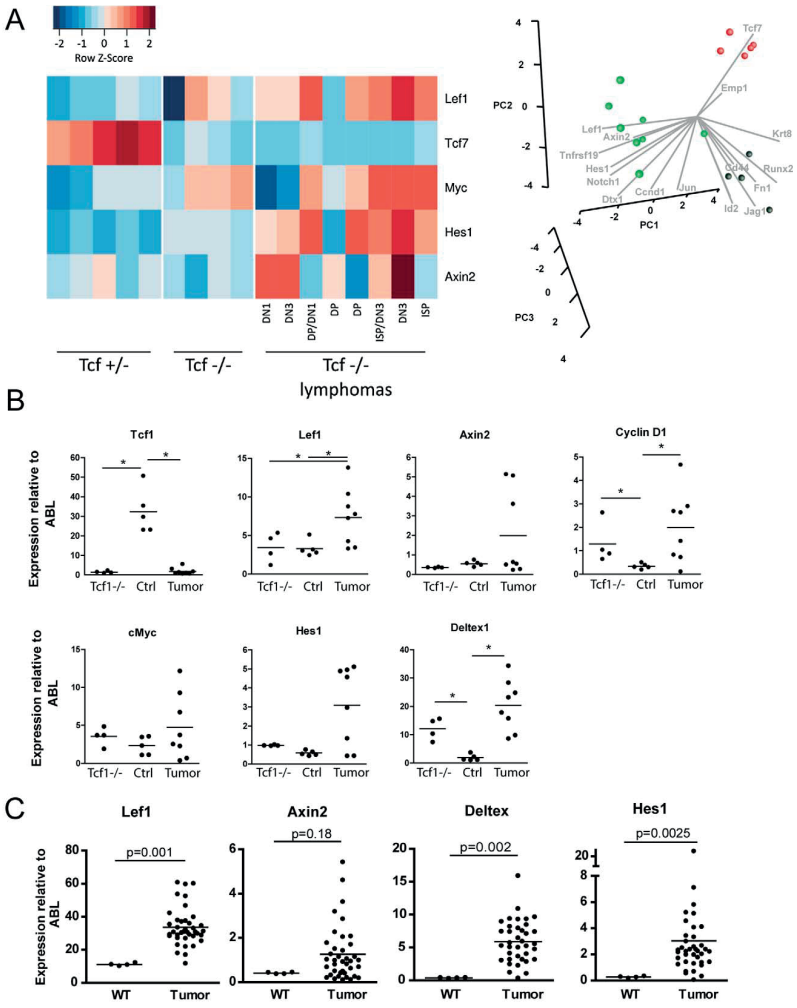

Figure 3. Tcf1$^{-/-}$ lymphomas show deregulated Wnt signaling.

(A) RNA isolated from thymi of 17 different mice was used for microarray analysis. Expression of Lef1, Tcf7, Myc, and Hes1 in the Tcf1$^{-/-}$ mice without lymphoma (n=4), Tcf1$^{+/-}$ mice (control, n=5) and Tcf1$^{-/-}$ mice with a lymphoma (Lymphoma, n=8) is shown. For the Tcf1$^{-/-}$ lymphoma mice, the phenotype of the tumor is indicated on the horizontal axis. Columns represent independent RNA preparations of the different mice groups. A principal component analysis was performed using Wnt and Notch target genes. A PCA analysis shows clustering of the three groups as well as the effect each of the target genes has on the separation of these groups with samples of Tcf1$^{+/-}$, Tcf1$^{-/-}$, and Tcf1$^{-/-}$ with tumors indicated by red, black, and green spheres, respectively. (B) Expression levels of Tcf1, Lef1, Axin2, Cyclin D1, cMyc, Hes1, and Deltex1 as determined by Affymetrix microarray were summarized and normalized using RMA, and the expression relative to Abl was plotted for each sample. Statistical significant differences (p<0.05) as determined by Mann–Whitney U test are indicated by an asterisk. (C) A panel of 40 Tcf1$^{-/-}$ thymic lymphomas and four control thymi (Tcf1$^{+/-}$) were analyzed by RQ-PCR. Expression data for Lef1 long (containing the β-catenin interacting domain), Axin2, Deltex1, and Hes1 are shown relative to the house keeping gene Abl. Mann–Whitney U tests were performed to calculate the indicated p values.

Principal component analysis of the Wnt target genes in all 17 thymic samples confirmed the obvious discriminating factor between Tcf1$^{-/-}$ and Tcf1$^{+/-}$ samples to be *Tcf7* (the HUGO gene name for Tcf1). The Tcf1$^{-/-}$ tumor samples were clearly distinguished by factors involved in the Wnt-signaling pathway, *Axin2*, *Lef1*, and *Tnfrsf19*, or in the Notch signaling pathway, *Deltex1* and *Hes1* (Figure 3A, right panel). These results indicated that both Wnt and Notch signaling are affected in the Tcf1$^{-/-}$ tumor samples compared to the other two groups. Tcf1$^{-/-}$ samples without a tumor were distinguished by low expression of the following factors: *Emp1*, *Krt8*, *Runx2*, *CD44*, *Fn1*, *Jag1*, *Id2*, and *Cdh1*. Several of these genes are known to be Wnt target genes (*Runx2*, *Id2*, *CD44*, and *Fn1*). These data show ectopic up-regulation of Wnt signaling as demonstrated by high expression of *Lef1*, *CyclinD1*, and *c-Myc* as well as Notch target genes *Hes*1 and *Deltex1* (Figure 3B). Collectively, these data indicate that interaction between the Wnt and Notch pathways is necessary for full lymphomagenesis.

Confirmation of the array data was performed with a panel of 40 Tcf1$^{-/-}$ thymic lymphomas by Q-PCR. In all tested tumor samples, the expression level of Lef1 was increased compared to thymocytes of control mice (Figure 3C). The mean expression Axin2 level of the 40 tumor samples was 4 times elevated compared to the mean expression Axin2 level of the control mice (1.2 versus 0.3), with 29 of the 40 tumor samples (73%) having a higher Axin2 level than 0.3 (Figure 3C). Moreover, the high *Axin2* expression in the majority (73%) of lymphomas in combination with the universally up-regulated Lef1 expression indicates a marked increase in Wnt signaling in these lymphomas. Further analysis of this panel of lymphomas showed that the expression levels of Hes1 and Deltex1, two target genes of Notch1 signaling, were enhanced in all tumor samples compared to the control samples (Figure 3C), again demonstrating that both the Wnt and Notch pathway are involved in full lymphomagenesis. As high Lef1 expression is already present in pre-leukemic samples (Figure 3A), it is likely that deregulated Wnt signaling predisposes thymocytes to induction of activating somatic mutations in Notch1, which subsequently accelerate lymphoma development.

Wnt-Reporter Activity Is Minimally Present in Normal Tcf1−/− Thymocytes and Enhanced in Tcf1−/− Lymphoma Cells

To confirm the paradoxical finding that mice lacking Tcf1 suffer from thymic lymphomas due to deregulated high Wnt signaling rather than low, we crossed Tcf1$^{-/-}$ mice with a well-established Wnt-reporter mouse strain, namely the Axin2-LacZ mice. Wnt-activity in these mice can be measured by the expression of β-galactosidase driven by the Axin2 promoter. In Figure 4A, the CD4/CD8 dot plots are shown of thymocytes of four different representative mice. The histograms show the Wnt-activity in DP, ISP, and DN3 cells for Tcf1$^{+/-}$ thymocytes (filled), Tcf1$^{-/-}$ thymocytes (thin line), and tumor Tcf1$^{-/-}$thymocytes (thick line). The thymocyte subsets of a Tcf1$^{-/-}$ control mouse without a tumor show severely reduced

Wnt-activity in ISP and DN3 thymocyte subsets compared to the $Tcf1^{+/-}$ control mouse (Mean Fluorescence Intensity [MFI] of 385 and 104 compared to 874 and 635 in control ISP and DN3, respectively), indicating a strongly diminished nuclear response to Wnt signals due to the Tcf1 deficiency. Interestingly, residual Wnt-activity can be measured in $Tcf1^{-/-}$ thymocytes, which suggests that Lef1 is mediating low levels of Wnt-activity in $Tcf1^{-/-}$ mice as a likely compensatory mechanism (as also shown by Figure 3A). $Tcf1^{-/-}$ mice developing lymphomas show enhanced Wnt activity in the developmental stages in which the tumor cells are blocked (MFI of 1,425 and 1,225 for Wnt-reporter signal in DP and ISP for tumor 1 and 2,123, 2,374, and 1,203 in DP, ISP, and DN3 for tumor 2). The thymi of the $Tcf1^{+/-}$ control mouse and the two $Tcf1^{-/-}$ tumor mice displaying high Wnt activity were further examined for the RNA expression levels of Lef1 and Hes1. The expression level of Lef1 was increased in both $Tcf1^{-/-}$ induced lymphomas, indicating that these high levels of Lef1 underlie the highly active Wnt signals in these tumors (Figure 4B). Interestingly, only in tumor 2 ($>175\times10^6$ cells) were high levels of the Notch target gene Hes1 observed, indicating that Notch signaling accelerates or maintains tumor development once it is initiated by deregulated Wnt-signaling. Indeed, when we compared the thymus size to the expression level of Hes1, we found that only in large $Tcf1^{-/-}$ tumors ($>25\times10^6$ cells) is the expression level of Hes1 increased (Figure 4C). These data suggest that a first oncogenic hit is the deregulation in Wnt-signaling due to high levels of Lef1 and that deregulation of the Notch-pathway is a secondary acquired mutation. To check for mutations in Notch1, we sequenced both the heterodimerization domain (HD), exon 26 and 27, and the PEST domain, encoded by exon 34. This analysis showed that in $Tcf1^{-/-}$ thymi (without tumor) and $Tcf1^{+/-}$ samples, no mutations were found in the three exons ($n = 8$, unpublished data). Analysis of the panel of 40 $Tcf1^{-/-}$ thymic lymphomas demonstrated mutations in exon 34 in all but one thymic lymphoma sample (unpublished data), which is known to promote Notch1 signaling by increasing the half-life of intracellular Notch, hence promoting tumor survival and growth. Together, these results suggest that Tcf1 deficiency leads to a pre-leukemic stage that favors additional mutations, most notably in Notch1.

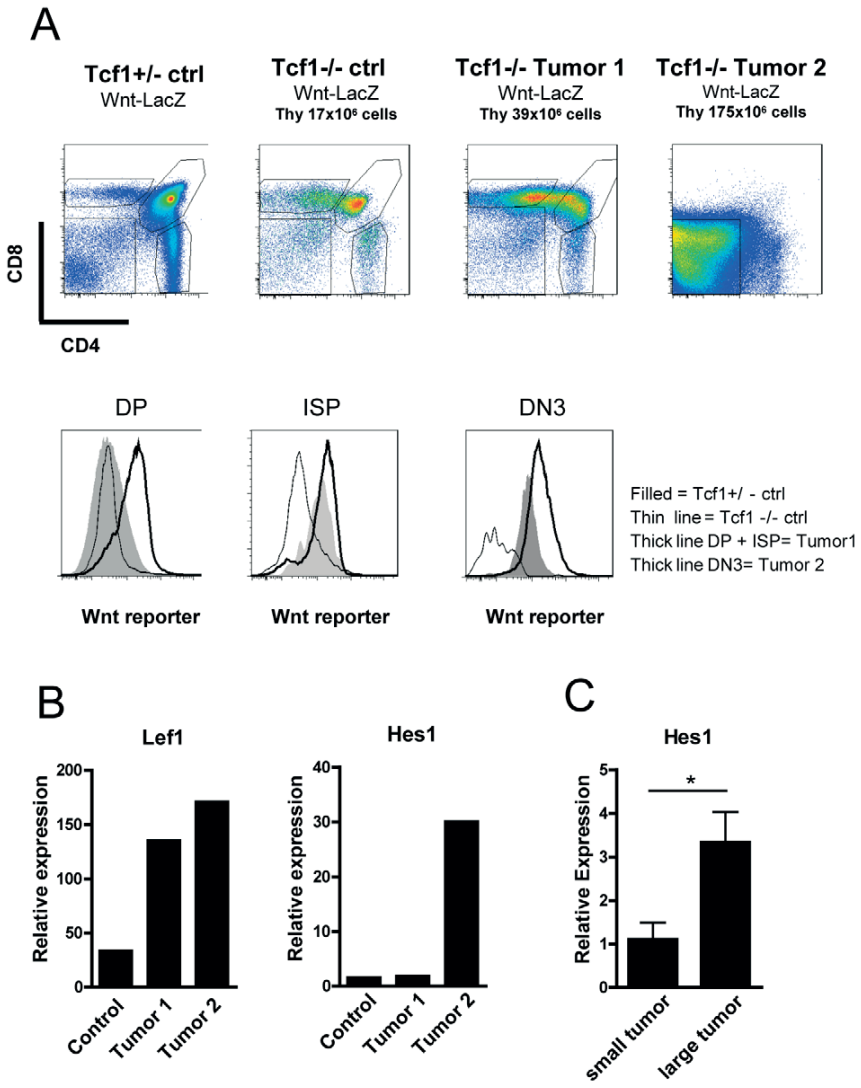

Figure 4. Blocked Tcf1$^{-/-}$ thymocytes show high Wnt-signaling in lymphoma development.

(A) Tcf1$^{-/-}$ mice were crossed to Conductin(Axin2)-LacZ reporter mice and sacrificed at different ages. Dot plots of four representative mice are shown: one control Tcf1$^{+/-}$ LacZ (age 20 wk), one control Tcf1$^{-/-}$ LacZ (age 9 wk), and two Tcf1$^{-/-}$ Axin2-LacZ mice (age 20 wk, Tumor 1+ Tumor 2). Organs were collected and thymocytes were stained for CD4, CD8, CD3, CD44, and CD25 together with FDG to demonstrate Wnt reporter activity in the different thymocyte subsets. The corresponding thymus sizes for the four mice shown are 110×10^6, 17×10^6, 39×10^6, and 175×10^6 cells, respectively. Expression of Wnt reporter activity is shown per thymocyte subset, DP, ISP, and DN3 for control cells (filled), Tcf1$^{-/-}$ cells (thin line), and Tcf1$^{-/-}$ tumor cells (thick line). (B) RNA was isolated of total thymus and the expression level of Lef1 and Hes1 relative to Abl is shown for the control thymus and the two tumor samples as shown in (A). (C) The mean expression levels of Hes1 relative to Abl are shown for small tumors (n=5) and large tumors (n=25).

Tcf1 Is the Major TCF/LEF Factor in the Thymus and Its Short Isoforms Function as Repressors of Lef1 Expression

To gain further insight into the mechanism underlying lymphomagenesis, the balance between the long and short isoforms of Lef1 and Tcf1 was investigated. It is known that the balance between the long and short isoforms of these factors is crucial in regulating Wnt signaling, as only the long isoform can bind β-catenin and hence mediate Wnt signaling, whilst the short isoform is considered the natural antagonist of Wnt signaling (a simplified version of the short versus the long form of Lef is shown in Figure 5A). Analysis of the $Tcf1^{-/-}$ tumor samples (RNA and protein) revealed that the normal ratio of long over short isoforms for Lef1 was altered in favor of the long isoform of Lef1, which mediates transcription of Wnt-β-catenin target genes (Figure 5A). The RNA levels of Tcf1 and Lef1 in normal thymus indicated a 10 times higher expression of Tcf1 than Lef1 in the thymus (Figure 5B). This is further illustrated by an example of the protein levels of Tcf1 and Lef1 in a nuclear extract protein sample of total thymocytes (Figure 5B, right panel). Reciprocal regulation of Tcf1 and Lef1 at the protein level was further examined in the major sorted thymic subsets (DN, DP, and SP). Of interest, the ratio between long Wnt responsive isoforms and short repressor isoforms is different for Tcf1 versus Lef1. For Tcf1, there is a clear expression of the inhibitory short isoforms in all thymocyte subsets, whilst for Lef1 in all stages the long β-catenin binding form is more abundant, except for the single-positive stage (Figure 5C). This suggests that major repressors of Wnt signaling in the thymus are formed by the Tcf1 short isoforms. Therefore, in the absence of Tcf1, a repression of Lef1 expression in the thymus is diminished. This allows for high levels of Lef1, which naturally occurs in a ratio of more Wnt responsive than inhibitory isoforms, hence strengthening the Wnt responsiveness of the (pre)leukemic cells. Thus, a major function of Tcf1 appears to control Lef1 expression via its short isoforms.

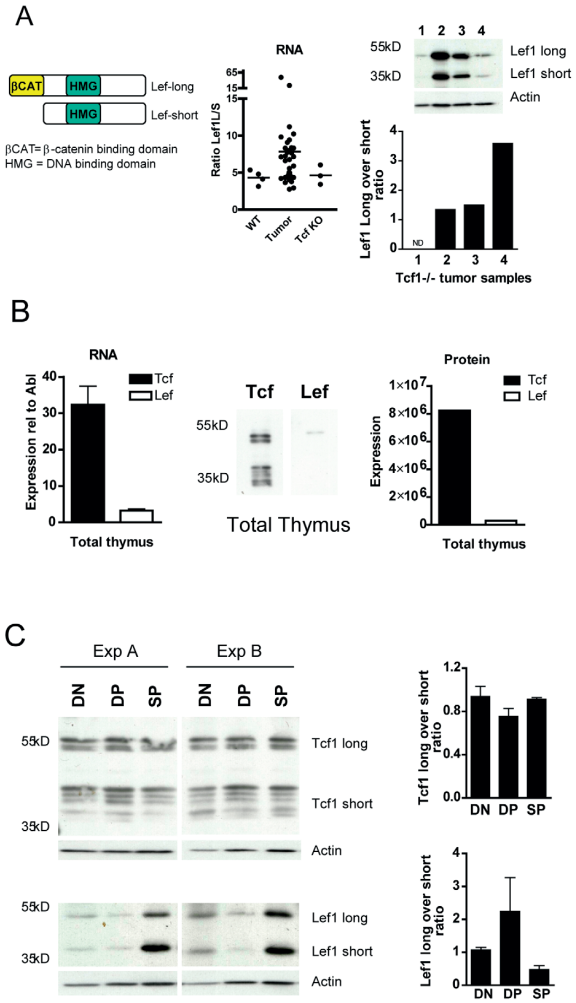

Figure 5. Lack of Tcf1 deregulates the balance between long and short isoforms of Lef1 in Tcf1-deficient lymphomas.

(A) A schematic representation of the long and short isoform of Lef1, depicting the β-catenin binding domain and the HMG-box (DNA binding domain). The ratio of Lef1 long isoform versus Lef1 short isoform was determined using RQ-PCR for 40 lymphomas, four control thymi, and three $Tcf1^{-/-}$ thymi without tumor. The long versus short ratio for Lef1 at the protein level is depicted for four different $Tcf1^{-/-}$ thymic tumors (tumor size: 19, 224, 118, 30×10^6 cells/ml, respectively). (B) Left panel, total Tcf and Lef RNA levels relative to Abl were determined using the data obtained from the Affymetrix data shown in Figure 3A ($Tcf1^{+/-}$ total thymus, n=5). In the middle panel, a Western blot analysis and quantification (right panel) for nuclear protein extracts of total thymocytes ($Tcf1^{+/+}$ mouse) is shown. (C) Representative Western blots of two independent experiments (Exp A and Exp B) of sorted thymocyte subsets from two control $Tcf1^{+/+}$ mice. Cell populations were sorted into the subsets DN, DP, and SP using lineage markers; CD3, CD4, CD8, CD44, and CD25 and total protein extracts were generated. Antibodies recognizing all isoforms of Tcf1 and Lef1 were used, and β-actin was used a loading control for the Western blots. Quantification of the Tcf and Lef signal of all Western blots was performed by ImageQuant J.

Deregulated Wnt Signaling Frequently Cooperates with Deregulated Notch Signaling in Tcf1–/– Induced Lymphomas

The data described above suggest that both deregulated Wnt as well as Notch signaling are required for development of the Tcf1-deficient lymphomas. To investigate the Wnt and Notch dependency, we performed a number of experiments with pharmacological drugs and genetic tools. Using the γ-secretase inhibitor DAPT, a potent Notch1 inhibitor, clear loss of survival and proliferation was observed in $Tcf1^{-/-}$tumor cells (Figure 6A, left figure). Moreover, the high Lef1 levels in the tumor cells appeared not to be a result of the deregulated Notch signaling, as inhibition of the $Tcf1^{-/-}$ tumor cells by DAPT only mildly affects Lef1 levels, whilst both Notch target genes *Hes1* and *Deltex* were completely down-regulated (Figure 6A, right figure). Ongoing activation of the Wnt signaling pathway was shown to be crucial for the survival of the $Tcf1^{-/-}$ tumor cells in two different sets of experiments. First of all, incubation of the generated $Tcf1^{-/-}$ cell lines with the Wnt inhibitor Quercetin, which blocks the interaction between β-catenin and Tcf/Lef factors, induces rapid cell death (7 h), whilst the non-Wnt-dependent Jurkat cell line was minimally affected (Figure 6B). In addition, transfection of a dominant negative form of Tcf1/Lef1 in $Tcf1^{-/-}$ lymphoma cells also induced rapid cell death (>80% cell death after 6 h; Figure 6C). Thus, both ongoing Wnt and Notch signaling are required for the survival of Tcf1-deficient lymphomas.

To further investigate the Wnt and Lef1 dependency, we performed reporter gene analysis using the natural Lef1 promoter, which contains four consensus Tcf/Lef binding sites (Figure 6D). The luciferase experiments with the Lef1 promoter show that this promoter is Wnt responsive and demonstrate that Lef1 expression can be up-regulated by β-catenin-Lef1 complexes, providing a positive feedback loop. Such a positive feedback loop has been suggested before [25]. Consistent with the Wnt responsiveness, transfection of a ΔN Tcf1 construct, which acts as a dominant negative competitor, was capable of abolishing the β-catenin-induced activation of Lef1-dependent transcription. Thus, the Lef1 promoter is Wnt responsive and negatively regulated by short Tcf1 isoforms that lack the β-catenin interaction domain. Taken together, these data indicate that the highly deregulated Wnt signaling in the tumor cells is driven by Lef1; that it is frequently associated with increased Notch signaling, which acts as a collaborative oncogenic event; and that it is continuously required for survival of these lymphomas.

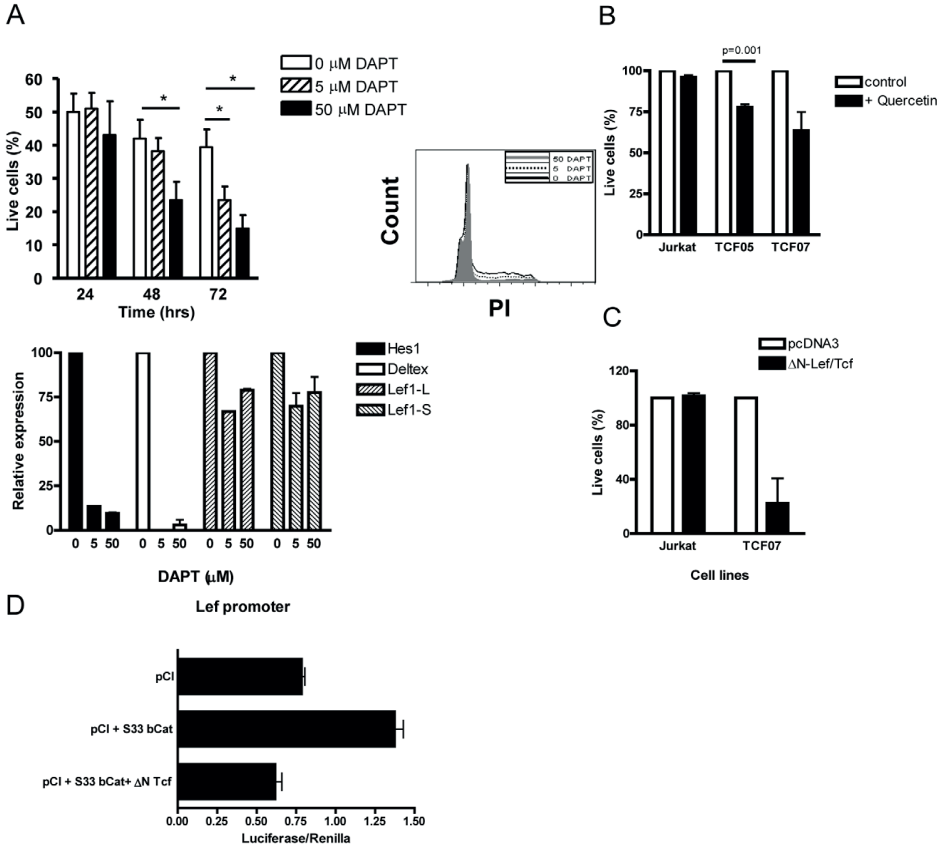

Figure 6. Tcf1-deficient lymphoma cells depend on Wnt and Notch signaling for their survival.

Several Tcf1$^{-/-}$ cell lines were established from Tcf1$^{-/-}$ thymic lymphomas, and all cell lines show Notch1 mutations and a high ratio of Lef1 long over short isoform. (A) The TCF05 cell line (phenotyped as an ISP tumor) was cultured in the absence and presence of the indicated concentrations of γ-secretase inhibitor (DAPT). Percentage of live cells was determined after 24, 48, and 72 h using flow cytometry (AnnexinV/7AAD staining). After 24 and 48 h cell cycle analysis was determined by propidium iodide staining, and the relative expression of Hes1, Deltex, Lef1-L, and Lef1-S was determined. For the cell cycle analysis, one representative example is shown and the mean expression levels relative to Abl are shown of two independent experiments. (B) Three cell lines (Jurkat, TCF05, and TCF07) were cultured for 7 h in the presence or absence of Quercetin (25 μM), and the percentage of live cells was determined by flow cytometry (7AAD/AnnexinV staining). The mean percentage (± SEM) of live cells (7AAD−/AnnV−) is shown for the three cell lines of three independent experiments. (C) The Tcf1$^{-/-}$ cell line TCF07 was established from a thymic lymphoma characterized as a DN3 tumor with a mutation in exon 34 of Notch1. This cell line and Jurkat cells were transfected with a GFP construct in combination with a control construct (pcDNA3) or a dominant negative Lef1/Tcf1 (pcDNA ⊿NTCF) construct (used ratio GFP:construct, 1:10). The percentage of viable cells was determined within the transfected (GFP+) cells after 6 h. (D) HEK 293 T cells were transfected with a LEF-1 luciferase reporter plasmid containing Tcf/Lef-responsive elements. The cells were cotransfected with S33-Bcat or pCI (3 μg) or Delta N Tcf1 together with S33 B cat. To control for transfection efficiency, all transfections included the pRLTK-renilla reporter (0.15 μg). Luciferase activities are shown as mean of three independent experiments normalized to renilla activity.

Discussion

During normal hematopoiesis, Tcf1 is required to induce and maintain proliferation of developing T cells in the thymus [19] and inhibit apoptotic signals at the DP stage [23]. Absence of Tcf1 not only results in a severely reduced thymic cellularity but also blocks differentiation of the thymocytes [11],[19],[21]. It is remarkable that $Tcf1^{-/-}$ cells blocked in differentiation develop into lymphoma cells, as we here report. We propose that $Tcf1^{-/-}$ blocked thymocytes give rise to lymphoma cells due to deregulated Wnt signaling, which is driven by expression of deregulated expression of Lef1. This model is based on several key observations (Figure 7). First of all, in essentially all $Tcf1^{-/-}$ tumors, a high expression of *Lef1* (selectively of the long form of Lef1, which is able to bind β-catenin) was found. This up-regulation of Lef1 likely acts as a compensatory mechanism for the lack of Tcf1 and is probably caused by lack of repression by Tcf1. We show much higher Tcf1 than Lef1 expression in the normal thymus, including the naturally occurring dominant negative isoforms of Tcf1. This suggests a direct repressor function of Tcf1 for Lef1 expression given the normally much higher Tcf1 than Lef1 expression in thymus. Especially since Lef1 has more long-beta catenin responsive isoforms (except in the SP stage), while Tcf1 has slightly more expression of the short form. Hence in the complete absence of Tcf1, Lef1 will take over as a Wnt-responsive transcription factor in the thymus. Thus, the lymphoma development is initiated by developmental arrest due to lack of Tcf1; thereby, the suppression of Lef1 expression by short Tcf1 isoforms is lifted. This leads to higher Lef1 expression and a propensity to higher Wnt responsiveness, restoring proliferation and increasing Wnt target gene expression and concomitantly to the possibility of induction of somatic mutations, such as those found in Notch1. Lef1 expression can be further enhanced by Notch, as shown in lymphomas that lack the E2A transcription factor [27]. Moreover, Lef1 can also positively regulate its own expression through Wnt dependent ([26], plus data in Figure 6D) and independent mechanisms [26],[28]. Finally, up-regulation of other oncogenes will lead to frank lymphoma/leukemia development. In this respect, up-regulation of T-ALL oncogenes such as *Lmo2* and *Mef2C* in the $Tcf1^{-/-}$ thymocytes may also contribute to the preleukemic nature of these cells. Importantly, the in vivo evidence for this model was provided by crossing Tcf mice with Axin2-LacZ-reporter mice. $Tcf1^{-/-}$ mice without tumors have a reduced level of Wnt-activity in all thymocyte subsets compared to $Tcf1^{+/-}$ mice, whilst $Tcf1^{-/-}$ thymic lymphoma cells show a very high level of Wnt-activity in blocked thymocytes.

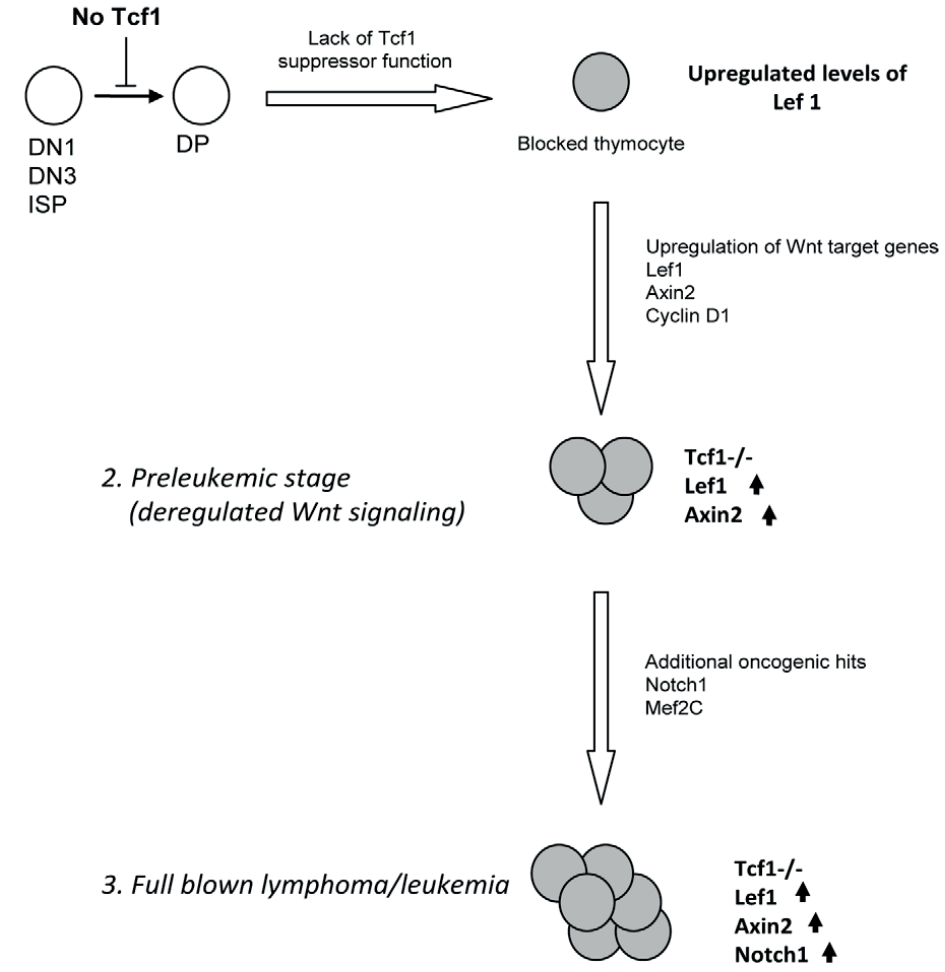

Figure 7. ***Mechanism of lymphomagenesis caused by Tcf1 deficiency.***

In the absence of Tcf1, thymocyte development is blocked at several stages (DN1, DN3, and ISP). Arrested thymocytes lack all isoforms of Tcf1, including the repressive isoforms. Loss of these repressive isoforms results in an up-regulation of Lef1, of which the long isoforms are most abundant in the thymus. Subsequently, Lef1 is capable of interacting with β-catenin as a compensatory mechanism, inducing deregulated Wnt signaling as measured by the high expression of Axin2 in the vast majority of the lymphomas, forming a pre-leukemic stage. After additional mutations are acquired, of which activating mutation in Notch1 frequently occur, and also other oncogenes such as Mef2C and Lmo2 are likely candidates to be affected, full-blown lymphoma/leukemia develops.

It is of interest to compare the Tcf1$^{-/-}$ lymphomas with two other murine lymphoma models, namely those induced by activated β-catenin and by lack of E2A. The development of Tcf1$^{-/-}$ lymphomas contrasts with thymic lymphomas induced by overexpression of β-catenin in that the latter show no Notch1 mutations [29] but may be dependent on p53 absence [30]. In the E2A-deficient lymphomas, Lef1 is a Notch target gene only in the context of the lymphoma cells, but not in normal progenitors, and Lef1 is essential for lymphomagenesis. Thus, in the absence of normal regulatory mechanisms in thymic lymphoma cells provided by E2A or Tcf1, Lef1 can act as an important oncogene. Interestingly, the Tcf1$^{-/-}$ thymic lymphomas easily gain additional mutations in the *Notch1* gene (in contrast to the β-catenin-dependent lymphomas), which leads to further development of these lymphomas. Once Notch1 expression is established, it may serve as an accelerator of the Lef1-mediated deregulated Wnt signaling, ensuring increased survival and expansion of the lymphoma cells [27].

In the human equivalent of these T-cell lymphomas, T-cell acute lymphoblastic leukemias (T-ALL), several genetic abnormalities have been described including Notch1 mutations in a large proportion of all human T-ALL [31]. While it is difficult to unravel the stepwise process of leukemia development in humans, the activating mutations in Notch1 are not always the initiating events as shown by data from a leukemia that was observed in a gene therapy trail for X-linked SCID [32]. In this case, it was conclusively shown that insertional mutagenesis near the LMO2 proto-oncogene was the first genetic aberration followed by Notch1 mutations and further genetic aberrations [32]. It will be of high interest to see if loss of Tcf1 tumor suppressor function occurs in human T-ALL. Whether loss of function of Tcf1 as a tumor suppressor gene actually occurs in human T-ALL is currently under investigation. Human T-ALL with mutations in Lef1 have been described, although the mechanistic consequence of these mutations is currently unclear [33].

Two recent studies from the Bhandoola and Gounari laboratories, respectively, have conclusively demonstrated a key role for Tcf1 in establishing T-cell commitment [10],[16].

Deletion of β-catenin in the thymus has been reported both to affect T-cell development (using Lck-CRE) or to have no effect at all, when using *Mx*-Cre-mediated deletion of β-catenin [34] or β- and γ-catenin simultaneously [35],[36]. However, our recent work on Wnt dosage in various hematopoietic lineages including thymocytes suggests that the lack of phenotype using mx-Cre might be caused by the fact that Wnt signaling was not completely abolished in these models [37],[38]. Moreover, the Held Group also published that the Tcf1 phenotype can be complemented by transgenic expression of a long Tcf1 isoform but not by a short (non/Wnt responsive) isoform [23]. Therefore, a major role for Tcf1 in the thymus is to integrate Wnt responsive signals and thereby allow T-cell development to occur normally. Nevertheless, our current work also indicates an important non/Wnt-

dependent role of TCF, as a negative regulator of the Wnt pathway. The recent work of Bhandoola is also interpreted as a non/Wnt-dependent effect. Therefore, it is an intriguing possibility that both Wnt-dependent effects of Tcf1 (e.g., Wnt-driven proliferation of DN thymocytes) and Wnt-independent effects (induction of T-cell specification) collaborate in the early stages of T-cell development.

In summary, we here report that Tcf1 has a dual function during T-cell development: first, it is needed as a transcriptional activator of Wnt-induced proliferation, but unexpectedly it also acts as a transcriptional repressor and tumor suppressor gene to prevent the development of thymic lymphomas and it may also function in a Wnt-independent way in these early stages of T-cell development, as suggested by recent work [16] perhaps by repressing genes of alternative (non-T) lineages. We conclude that Tcf-1 acts as a molecular switch between proliferative and repressive signals during T-cell development in the thymus.

Materials and Methods

Mice

C57Bl/6 Tcf1$^{-/-\ \Delta VII/\Delta VII}$ were originally described by Verbeek [19], C57Bl/6-CD45.1 (Ly5.1) and C57Bl/6-Rag1$^{-/-}$ mice were obtained from the Jackson Laboratory, and Conductin(Axin2)-LacZ mice were kindly provided by B. Jerchow and W. Birchmeier (Max Delbrück Center for Molecular Medicine, Berlin, Germany) [39]. All mice were kept in the specified pathogen-free (SPF) breeding section, and this study was approved by the institutional Animal Ethical Committee of the Erasmus MC, Rotterdam and the Leiden University Medical Center, Leiden.

Immunohistochemistry

Paraffin sections of organs of Tcf1$^{-/-}$ mice were stained with H&E or with antibody against CD3 (A045229; DAKO, Glostrup, Denmark) and biotinylated goat anti-rabbit IgG (BA-1000; Vector Labs, Burlingame, CA, USA) as the secondary antibody. Visualization was enforced with ABC staining kit (PK6100, Vector Labs) with 3,3'-diaminobenzidine tetrahydrochloride (DAB, D5637, Sigma-Aldrich, St Louis, MO, USA) as substrate. Mayer's hematoxilin was utilized as nuclear counterstaining.

Flowcytometry

The following antibodies were obtained from BD Biosciences (San Diego, CA): anti-CD3-APC (145-2C11), anti-CD4-PeCy7 (RM4-5), anti-CD8-PerCP(53-6.7), anti-CD25-PE (PC61), anti-CD44-PE (IM7), anti-CD24-FITC (M1/69), anti-cKit-PeCy7 (2B8). Lineage markers Mac1 (M1/70), Gr1 ((RB6-8C5), B220 (Ra3-6B2), and Ter119 and Nk1.1 (PK136) were all biotinylated and streptavidin APC-Cy7. Cells were stained in Fluorescence-activated cell sorter (FACS) buffer (PBS, 2% bovine serum albumin, 0.1% sodium azide) for 30 min at 4°C. Intracellular β-galactosidase activity was measured by staining cells with 2 mM fluorescein di-β-D-galactopyranoside (FDG) substrate (Molecular Probes). FDG was loaded into the cells by hypotonic shock at 37°C for 1 min, prior to cell surface antibody staining. The β-galactosidase reaction was stopped with 1 mM phenylethyl β-D-thiogalactopyranoside (PETG, from Molecular Probes). Cells were washed and immediately analyzed on a Canto I (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

In Vivo Tumor Induction

Lymphoma or thymocyte suspensions of previously characterized mice were prepared aseptically. Cells (5×10^5) were injected in the tail vein of sublethally irradiated (4 Gy) Rag1$^{-/-}$ mice. The mice were bled every 4 wk until the end of the experiment. The presence of lymphoma cells was investigated by flow cytometry.

Southern Blot Analysis

DNA (10 µg) was digested with *Eco*RI overnight at 37°C, separated on a 0.7% agarose gel, and blotted onto a positively charged nylon membrane (Hybond, Amersham). Southern blots were probed with a ^{32}P-labeled 1.2 kb *Eco*RI-*Cla*I genomic fragment recognizing the Jβ2 region of the TCRβ gene.

Western Blot Analysis

Total protein lysates and nuclear extracts were generated from total thymocytes and sorted populations (DN, DP, and SP). Total protein lysates were generated by immediately lysing the cells in boiling sample buffer (150 mM Tris-HCl pH 6.8, 300 mM DTT, 30% glycerol, 6% SDS, 0.1% bromophenol blue). Nuclear extracts were generated by resuspending cells in buffer A (10 mM Hepes, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 0.5 mM DTT and freshly added protein inhibitor cocktail [PIC]) for 15 min on ice. Subsequently NP40 (final concentration 0.6%) was added, thoroughly mixed, and the cytoplasmic extract was removed by centrifugation. Remaining nuclei were lysed by incubating with buffer C (20 mM Hepes, 10% glycerol, 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, and freshly added DTT and PIC) for 30 min at 4°C. Nuclear extracts were ready after centrifugation. Protein concentration was measured using BCA Protein Assay kit (Pierce, Rockford, MD, USA). Lysates containing 1 µg of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Nonspecific binding was blocked by incubation in blocking buffer (2.5% BSA in TBS-Tween) followed by incubation with the primary antibodies and the appropriate secondary antibodies conjugated to horseradish peroxidase. All isoforms of Tcf1 were detected by anti-Tcf1 antibody (clone C46C7, rabbit mAb, Cell Signaling, Boston, USA), and all Lef1 isoforms were detected by anti-Lef1 antibody (clone C18A7, rabbit mAb, Cell Signaling). Equal loading was confirmed by reprobing the blots with an anti-actin antibody.

Microarray Analysis

Thymocytes were homogenized for RNA isolation using Qiagen RNeasy minicolumns. The quantity and quality of total RNA was determined using spectrophotometry (Nanodrop) and an Agilent Bioanalyzer. One µg of RNA was used to generate cRNA using Affymetrix One cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA, USA), after which the samples were biotinylated using an Affymetrix IVT labeling kit (Affymetrix). The samples were hybridized overnight at 42°C to GeneChip mouse genome 430 2.0 Arrays (Affymetrix). Washing and staining steps were performed on a Fluidics station 450, and the Genechips were scanned using a GeneChip scanner 3000 (Affymetrix) at the Department of Immunology, Erasmus Medical Center. Raw data were normalized and summarized using Robust Multichip Average (RMA) method [40]. Array analysis was performed using R-2.14 (http://cran.r-project.org/) and Bioconductor 2.9 software (http://www.bioconductor.org/) using the bpca [41] and gplots [42] packages. From the dataset, genes were selected for display in heatmaps,

in which the rows of the expression matrix were ordered by hierarchical clustering of Eucledian distances between the samples, with the expression intensities being scaled per probeset. Principal component analysis was performed on a dataset of Tcf1$^{+/-}$, Tcf1$^{-/-}$, and Tcf$^{-/-}$ tumor samples, using selection of Wnt and Notch response genes (*Emp1*, *Tcf7*, *Tnfrsf19*, *Hes1*, *Dtx1*, *Notch1*, *Axin2*, *Lef1*, *Cd44*, *Runx2*, *Fn1*, *Cdh1*, *Jun*, *Ccnd1*, *Krt8*, *Id2*, and *Jag1*). The first three principal components are displayed.

Real-Time Quantitative-PCR Analysis (RQ-PCR)

Total RNA was extracted using Qiagen RNeasy minicolumns. One µg of total RNA was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), Oligo dT, and random hexamer primers. The RQ-PCR reaction was performed using TaqMan Universal mastermix (Applied biosystems, Foster City, CA, USA) and was run on a PRISM 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). The following primers were used in combination with FAM-labeled probes from the universal probe library (Roche): Deltex1 forward primer: *5′-GAAGAACTTGAATGGCACTGG-3′*; reverse primer: *5′-GTTTGGGTGCTCGTGTCAG-3′*; Lef1 short forward primer: *5′-GCGACACTTCCATGTCCAG-3′*; reverse primer: *5′-TCCTGTTTGACCTGAGGTGTTA-3′*; Lef1 long forward primer: *5′-TGGTTAACGAGTCCGAAATCA-3′*; reverser primer: *5′-AGAGGACGGGGCTTGTCT-3′*; Axin2 forward primer: *5′-GCAGGAGCCTCACCCTTC-3′*; reverse primer: *5′-TGCCAGTTTCTTTGGCTCTT-3′*; Hes1 forward primer: *5′-AAACACTGATTTTGGAGCACT-3′*; and reverse primer: *5′-TGCTTCACAGTCATTTCCAGA-3′*. RQ-PCR results were normalized to *Abl* expression in the same sample: forward primer: *5′-TGGAGATAACACTCTAAGCATAACTAAAGGT-3′*; reverse primer: *5′-GATGTAGTTGCTTGGGACCCA-3′*; and probe: *5′-FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA-3′*.

Notch1 Mutation Analysis

cDNA of total thymus was used for the amplification of exons encoding the Notch1 heterodimerization and PEST domains. Primers used for the identification of activating Notch1 mutations are described elsewhere [43].

Tcf1–/– Cell Line Experiments

Several Tcf1$^{-/-}$ cell lines were established from Tcf1$^{-/-}$ thymic lymphomas, and all cell lines show Notch1 mutations and a high ratio of Lef1 long over short isoform. Transfection experiments were performed by transfecting the cell line with eGFP together with either a control construct (pcDNA3) or a dominant negative Lef1/Tcf1 construct (transfection ratio GFP:construct, 1:10) using AMAXA electroporation technology. Transfected cells were identified based on GFP positivity and phenotype, and cell viability was determined 6 h after transfection. Discrimination between viable and dead cells was performed by staining the cells with AnnexinV and 7AAD (BD Bioscience). Tcf1$^{-/-}$ cell line cultures were performed in

the presence and absence of the γ-secretase inhibitor DAPT (0, 5, and 50 µM) or Quercetin (50 µM). At the indicated time points, cell cycle analysis was performed using propidium iodide, live cells were determined by 7AAD/AnnexinV stain, and RNA was isolated for gene expression levels.

Luciferase Reporter Gene Assays

293 T cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, L-glutamin, and penicillin/streptomycin and transfected using the Fugene method according to the manufacturer's procedures (Roche). The cultures were transfected with 1.5 µg LEF-1 4000 luciferase reporter plasmid (containing four Tcf/Lef-responsive elements) or 1.5 µg of the LEF-1 600 luciferase reporter plasmid (all Tcf/Lef-responsive elements deleted) (kindly provided by Dr. J. Skokowa, Hannover Medical School [44]). The cells were cotransfected with S33-βcatenin and/or pCI and/or ΔN-Tcf (3 µg). To control for transfection efficiency, all transfections included the pRLTK-renilla reporter (0.15 µg). Transfected cells were cultured for 24 h and then lysed and assayed for reporter activity. Luciferase and Renilla activity was measured using a dual-luciferase reporter assay system from Promega (Madison, USA). All luciferase activities were normalized to Renilla activities.

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney U test (Prism GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

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CHAPTER 7

General Discussion

A precisely controlled balance between self-renewal and differentiation of hematopoietic stem cells (HSCs), is required to maintain the homeostasis of the hematopoietic system and to combat against stress situations like injury and infection efficiently. This balance is regulated by signals stem from the BM and the thymic microenvironments throughout the hematopoiesis and thymopoiesis. It has been a long-standing challenge to mimic the niche signals in vitro, with the aim to proliferate and manipulate HSCs and/or thymocytes for clinical purposes. The possibility of stimulating these cells with signaling ligands such as Wnt and Notch ligands is a very attractive approach since this would result in a more controlled therapy, compared to approaches requiring permanent gene modification. In fact, several reports have shown that Wnt ligands could efficiently induce the expansion of hematopoietic progenitors in vitro while maintaining an immature phenotype and/or repopulating capacity [1-4]. This would ultimately result in a faster thymic reconstitution by thymic seeding cells. However, most of these studies focus on canonical Wnt signaling, therefore the role of non-canonical Wnt signaling in this process has largely remained unknown. In this thesis, we attempted to have a side by side investigation of canonical and non-canonical Wnt singling during hematopoiesis and thymopoiesis.

Loss of stemness in HSCs with high levels of Wnt signaling

Previous work from our laboratory demonstrated that Wnt signaling is regulated in a dose-dependent manner during hematopoiesis and thymopoiesis [5]. Various Apc-mutant mouse models have been used to induce differential levels of Wnt signaling activation in vivo. It was shown that mild and intermediate levels of Wnt signaling regulate myeloid development, whereas, intermediate level regulates T cell development, but B-cell development is not controlled by Wnt signaling levels [5]. Apc encodes for a multifunctional protein responsible for various cellular functions [6]. However, most Apc mutant mouse models in which tumors are developed, depend completely on Wnt dosage.

Consistently, It has shown by other laboratories that high Wnt levels in HSCs eventually results in loss of stemness and lack reconstitution after total body irradiation of recipients [7-9]. In chapter 2, we used gene expression profiling to investigate the mechanisms underlying loss of repopulating capacity in HSCs with high levels of Wnt signaling. We showed at both genetic level as well as functional assays that HSCs with high levels of Wnt signaling activity have enhanced differentiation and decreased proliferation, without any effect on apoptosis. The much stronger tendency of these cells for differentiation towards mature blood lineages, associated with the loss of HSC proliferation is thought to be causative for lack of reconstitution. This observation could explain the lack of maintaining bona fide stemness in Apc exon 15 deleted HSCs.

These data are in agreement with another study described in chapter 4 in which activation of Wnt signaling by recombinant overexpression of Wnt3a resulted in a dose-dependent impact on murine HSC biology. Consistently in humans, it has been shown that treatment with very high levels of Wnt3a leads to loss of HSC proliferation in vitro [10]. It is worth to mention that also in the intestine, Wnt signaling induces maturation of Paneth cells, a mechanism in which active β-catenin and Tcf4 are involved [11], indicating that high Wnt signaling levels can lead to increased differentiation. Thus, Wnt signaling levels need to be strictly controlled when considering Wnt ligand as HSC expansion factor to be used for clinical purposes. It is plausible that intermediate Wnt levels, which has disadvantages for stemness status of HSCs, can be tolerated with increased HSC survival, which then would cause better self-renewal at this somehow higher Wnt signaling dose. It is well documented that PI3K/Akt signaling [12], as well as ectopic expression of Bcl2 [2], can provide such signals. Apparently, higher Wnt signaling levels can be endured in HSCs in the case of co-activation with other survival pathways. Interestingly, the high Wnt levels in combination with oncogene activation in acute myeloid leukemia seem to allow the Wnt pathway to function as a self-renewal factor for leukemic stem cells [13], whereas high Wnt levels cannot do so in normal HSCs. The different anatomical sites of healthy versus malignant HSCs in the bone marrow niche [14] might contribute to this discrete effect of high Wnt dosage and opens up a therapeutic window targeting leukemic but not normal stem cells.

Role of Ryk in HSC repopulation

In chapter 3 we studied the role of Non-canonical Ryk receptor in hematopoiesis and thymopoiesis. Our data indicate that Ryk as a co-receptor has a marginal role in hematopoiesis, possibly due to a redundant role with other tyrosine kinase receptors including Ror [15, 16] and/or Wnt signaling receptors and co-receptors such as FRZ 2 [17, 18] or FRZ8 in combination with Flamingo and LRP-5 and LRP-6 [19]

Previous reports suggest that the role of Ryk, similar to other receptors, is context and tissue dependent [15, 20]. Most probably, this is defined by several factors, including dominant expression of specific ligands in various tissues, or the expression of discrete co-receptors by surrounding cells. It has also been shown that Ryk can bind to both the Wnt3a canonical ligand [21], and Wnt5a non-canonical ligand [22] depending on the context and type of tissue, suggesting that different experimental settings might result in different outcomes. Our data in chapter 3 did not support an important function for Ryk during T-cell development as no differences between mutant and control littermates were observed in vitro in presence of both the Wnt3a and Wnt5a ligands. Nevertheless, the secondary

transplantation revealed that Ryk plays a role in stem cell repopulation. Transplanted Ryk KO stem cells are more apoptotic, while more proliferative compared with wild-type cells. Nemeth and co-workers have suggested that Ryk, via Wnt5a binding, can prevent the proliferation of LSK cells [23, 24]. In these reports, the role of Ryk in hematopoiesis has been investigated by using anti-Ryk polyclonal antibodies that presumably block the receptor [21, 22]. These researchers demonstrated that by adding polyclonal antibodies to the Ryk receptor a modest reduction in cell cycle at G0 phase (from 29 to 22%) occurs, which was interpreted as a deficiency effect, in which blocking Ryk would augment proliferation; this is consistent with our observation in which Ryk KO stem cells are more proliferative. However, polyclonal antibodies have a variable impact and could act as both activator and inhibitor antibodies. In addition, structural and functional studies have shown high levels of redundancy between Ryk and other tyrosine kinase receptor family members, in particular the ROR non-canonical Wnt receptors, making the study of each receptor specifically sophisticated [15]. A clear loss-of-function model as we employed in chapter 3 allows a more direct interpretation of the Ryk's function during hematopoiesis, although molecular redundancy by the associated Ror receptors could also play a role, and could be considered as a potential explanation for the marginal effect observed.

The impacts of treatment with polyclonal Ryk antibodies on long-term hematopoietic reconstitution were similar to our results, that is, reduced reconstitution in Ryk deficient stem cells. Therefore, loss of Ryk in HSCs decreases their quiescence, resulting in proliferation-induced apoptosis and diminished self-renewal. The combined increases in apoptosis and loss of quiescence in HSCs, likely explain the lower self-renewal of Ryk KO LSK cells.

The roles of canonical and non-canonical Wnts and potential cross-talk between the pathways, undoubtedly require more investigation, particularly, as Wnts are being used in stem cell expansion protocols [25, 26] including those employing designer nucleases for therapeutic gene editing [27]. Increasing evidence suggests the deregulation of both canonical and non-canonical Wnts in hematological malignancies. Previous work from Luis et al in combination with the data described in chapter 3 indicate that the dosage of canonical Wnt signaling is crucial in determining the functional outcome on reconstitution capacity of stem cells [5, 28]. Studying Wnt proteins for HSC expansion will require good in vivo reporter systems and well-defined reagents, taking the impact on apoptosis as well as on cell proliferation into account.

Distinct functions of Wnt3a and Wnt5a signaling in lymphopoiesis and thymopoiesis

Although binding of most Wnt proteins can trigger both canonical and non-canonical signaling pathways [29], some Wnt ligands (Wnt1, Wnt3A, and Wnt8) have been related to canonical signaling, whereas others (Wnt5A and Wnt11) are linked to non-canonical signaling. Induction of non-canonical Wnt inhibits the canonical pathway, partly because of competition for receptor binding, and partly by influencing intracellular β-catenin levels. Elegant studies from the Suda laboratory have indicated that non-canonical Wnt signaling is required to maintain HSC quiescence. Together with Flamingo, Fz8 is a non-canonical Wnt receptor that interacts with non-canonical Wnt produced by osteoblastic cells in the niche during homeostasis. However, canonical Wnt signaling is activated in HSCs to induce self-renewal and differentiation under stress conditions [19]. Indeed, non-canonical Wnt5a signaling is enhanced in short-term HSC repopulation to retain HSCs in a quiescent G0 state [24]. Recent reports associated with aging of the hematopoietic system have also indicated the significance of Wnt5a in the regulation of HSC biology. Aged HSCs are defined by decreased repopulation capability per HSC, increased myeloid output, and reduced lymphoid offspring (reviewed by Geiger et al.) [30]. In aged HSCs, Florian et al. showed a shift from canonical to non-canonical Wnt signaling as a consequence of enhanced expression of Wnt5a [31]. Treatment of young HSCs with Wnt5a immediately led to an aged HSC phenotype, with lowered repopulation capability and a myeloid differentiation tendency via induction of the small Rho GTPase Cdc42. Our data in chapter 4 described the effects of overexpression of the canonical Wnt3a and non-canonical Wnt5a in HSCs by lentiviral vector transduction of LSK cells from fetal liver, following by transplantation of the transduced LSKs into irradiated recipients [32]. Wnt3a overexpression led to increased B lymphopoiesis and fewer myeloid cells, whereas Wnt5a augments myelopoiesis in the bone marrow (BM) and spleen. Thus, canonical and non-canonical Wnt signaling have discrete roles in hematopoiesis, similar to other developmental systems [33]. We concluded that Wnt signaling affects cell fate decisions in HSCs; canonical Wnt signaling is vital for self-renewal and lymphoid developmental potential, whereas non-canonical Wnt signaling regulates quiescence and short-term myeloid offspring.

Wnt1 and Wnt4 have been shown to function as growth factors for DN cells, thus they are important for thymocyte proliferation [34] as Wnt1 and Wnt4 KO mice exhibit low thymic cellularity. Consistently, overexpression of Wnt4 has shown to selectively induce expansion of thymic output from transduced HSCs. Previous work from our laboratory showed that Wnt3a plays a critical role in fetal thymopoiesis, as Wnt3a KO thymi exhibit diminished numbers of DP cells due to an arrest at the ISP stage [35].

On the other hand Liang et al. identified that non-canonical signaling induces apoptosis in fetal thymic cells. They showed that exogenous Wnt5a is responsible for the induction of apoptosis in DP stage, while its deficiency diminished the PKC activation and simultaneously reduced the activity of CamKII [36]. In chapter 4 we performed a side by side comparison of the impact of Wnt3a canonical and Wnt5a non-canonical pathways during T cell development. We demonstrated that the activation of canonical Wnt signaling via Wnt3a is crucial for T cell development, and functions in dosage dependent fashion. While intermediate to low doses of canonical Wnt signaling would accelerate thymopoiesis, higher doses support B lymphopoiesis in vivo. On the other hand, Wnt5a non-canonical Wnt signaling triggers myelopoiesis in vivo and it does not function in a strict dosage dependent fashion. Additionally, Wnt5a signaling activation results in apoptosis in developing thymocytes in vitro.

These data are consistent with the previous studies in our laboratory in which we showed that canonical Wnt signaling functions in a dosage dependent fashion by using Apc hypomorphic mutations [5]. Although the Apc hypomorphic mutations proved to be a faithful model to alter canonical Wnt signaling in the hematopoietic system, it lacks the feedback mechanisms operating during normal thymopoiesis. In fact, control of Wnt signaling at the intracellular level is sophisticated because of multiple interactions with other pathways, including Notch and Hedgehog pathways. In the study described in chapter 4, we used Wnt3a, a natural ligand of canonical Wnt signaling, and we obtained identical data. This suggests that Wnt3a triggers canonical Wnt signaling via β-catenin and TCF/LEF dependent pathway in the thymus, [37] and it is possible to manipulate canonical Wnt signaling via differential concentration of Wnt3a in vivo. It is very likely that in the thymus, developing thymocytes are exposed to different types and concentration of Wnt ligands. Another possibility is that thymocytes express different FZD receptors with various binding affinity to the secreted Wnt proteins within the thymic microenvironment. Previous gene expression profiling in our laboratory proposed that this might be the case [38], although experimental proof awaits the development of specific antibodies for each Frizzled receptor suitable for flow cytometry. As a consequence, developing thymocytes would undergo different levels of Wnt signaling due to the accumulation of different amounts of β-catenin proteins in the cytoplasm.

Crucial role of Tcf-1 as a lineage fidelity factor during T cell development

Precise sequential analysis of B cells and T cell developmental pathways reveals that both pathways are very similar in several aspects including the proliferation of progenitors, lineage restriction process, immune receptor rearrangements and checkpoints for a pre- and

mature immune receptors. In contrast to B cell development, exact roles and relationships of transcriptional factors T cell development have remained largely unknown. This is mainly due to the fact that several transcription factors such as Tcf-1, Gata-3, and Bcl11b are involved during the process of T cell commitment and most of the studies have only focused on one of them separately [39-48].

Our results in chapter 5 show that Tcf-1 transcription factor functions as a lineage fidelity factor during T cell development. Tcf-1 deficiency results in the partial block during early stages of T cells development in particular during T cell commitment stages. We also showed that DN3 cells lacking Tcf-1 that supposed to be fully committed T cells have promiscuous gene expression and differentiate into DN-1 like cells, as well as cells from non-T cells lineages, including B cells and myeloid cells. In fact, crucial transcription factors that drive alternative lineages such as Bcl11a, Pax5, and Pu.1 are highly expressed in Tcf1-KO DN3 and DN4 cells, whereas in wild-type cells these factors are almost absent in these stages. This observation is similar to the findings of Dr. Busslinger and his coworkers on the role of Pax-5 during B cell development. They showed that fully committed B cells that lack Pax5 de-differentiate to multipotent progenitor cells that can replenish all hematopoietic lineages, even in vivo [49, 50].

Our data together with other studies have shown that Bcl11b and Gata3 are important target genes of Tcf1 [39, 47]. Therefore, we re-introduced these transcription factors in Tcf1 deficient cells to see if they could rescue the thymic phenotype. Gata3 overexpression in Tcf-1 KO stem cells suppressed B cell fate but also myeloid fate in the thymus. Interestingly, in the Bone marrow, myeloid suppression was not observed, but the effect on B cell development was also seen outside the thymus. Reversely, overexpression of Bcl11b promotes T cell commitment without affecting alternative lineage development in the thymus, resulting in a bypass of T cell developmental block caused by the lack of Tcf-1.

Therefore our data obtained in chapter 5 indicate a gene network in which Notch signals via RBP-JĶ launch the expression of Tcf1 which in turn activates Gata3 and Bcl11b, most likely in collaboration with Notch signals that can also directly act on these promoters. Interestingly, in skin stem cells, lineage infidelity predisposes to malignancy [51]. As Tcf1 deficiency leads to rapid development of T cell lymphomas (discussed in chapter 6), lineage infidelity may be a previously unrecognized factor in leukemogenesis, also in humans.

Tcf-1 as a tumor suppressor during lymphoma development

Our data in chapter 5 as well as other studies have demonstrated crucial roles for Tcf1 in T cell development and Lef1 in B cell development [52, 53]. Tcf1 KO mice have a severe reduction of thymic cellularity and multiple partial blocks at DN stages of thymocyte development, and eventually at the transition from the CD8+ ISP stage to the CD4+CD8+ double positive stage. ISP and DN subsets of Tcf1 KO mice do not proliferate as strongly as their wild-type counterparts [44]. Although Lef1 KO mice have a normal T cell development, Lef1 and Tcf1 double KO mice have a complete block at the ISP stage, which implicates redundancy between these two transcription factors [54]. Our study in chapter 6 demonstrated that Tcf1 has another essential function in the thymus in addition to functioning as the nuclear effector of Wnt signaling in thymocytes, namely a role as a tumor suppressor gene for the occurrence of thymic lymphomas, the murine counterpart of human T cell acute lymphoblastic leukemia (T-ALL). Tcf1 KO mice develop thymic lymphomas with high frequency at older ages due to ectopic upregulation of Lef1 and, paradoxically, extremely high Wnt signaling levels that initiate leukemia development [46], which is often followed by additional oncogenic hits such as Notch1 mutations.

Xu et al. have shown that β-catenin mediated Wnt signaling is required for the T-cell development to bypass the β-selection checkpoint using T cell specific deletion of β-catenin which ultimately results in diminished splenic T cells [55]. Furthermore, they have demonstrated that pre-TCR induced signals stabilize β-catenin via the activation of Erk [56]. Disruption of the β-catenin/Tcf and Lef interaction by using the inhibitor of β-catenin (ICAT) leads to Block at DN stages but not at later DP stages indicating a crucial role of Wnt signaling during DN to DP transition [57]. After allogeneic HSC transplantation in adults, development of the T-cell lineage is slow and inadequate. In fact, lack of proper thymic reconstitution results in a reduction in T-cell development and eventually hampers the diversity of T-cell repertoire [58, 59]. Interestingly, Shen et al. have shown that the activation of Wnt signaling by inhibiting GSK-3β with 6-bromoindirubin 30-oxime (BIO) affected the naive T-cell due to the decreased T cell differentiation [60]. Our data in chapter 6 indicates that the development of thymic lymphoma in Tcf1 KO mice is due to the activation of canonical Wnt signaling via Tcf1 and Lef1. Tcf-1 functions as a tumor suppressor, and a transcriptional repressor to inhibit the development of thymic lymphomas by suppression of genes of alternate (non- T) cell lineages. Therefore, Tcf-1 acts like a molecular switch between the proliferation of early thymocytes and repression of non-T cell development. Tcf-1 is also activated by Notch signaling and lack of Tcf-1 impairs the expansion of early T-cell progenitors which is most probably a Wnt independent effect [39]. Last but not least, Martins et al recently suggested that natural cell competition between young BM-derived and old thymic progenitors is crucial for inhibition of T-ALL development [61] Indicating

that thymic progenitor fitness is necessary for normal T cell development. This could be facilitated by tight regulation of Notch and Wnt signaling.

Deregulation of Wnt signaling in leukemia

Many studies implicate the deregulation of Wnt signaling in leukemia and tumors development. Gene expression analysis in various myelodysplastic syndrome (MDS) has revealed deregulation of cell cycle pathways and involvement of Wnt signaling and thrombopoietin [62]. A commonly deleted region in most of the MDS patients is 5q region which contains the APC binding domain. Consistently, It has been shown that deletion of APC in mice induces HSC deficiency and lack of reconstitution [63]. Acute myeloid leukemia (AML) stem cells have higher intracellular β-catenin level relative to the normal cells [64]. Chronic myelogenous leukemia (CML) cells undergo a higher level of Wnt signaling activity which is crucial for their growth [65]. Elevated levels of nuclear β-catenin were observed in Granulocyte–Macrophage progenitors in CML patients leading to higher self-renewal capacity [65]. Methylation of APC region in combination with the overexpression of β-catenin is also observed in a number of T-cell Acute Lymphoblastic Leukemia (T-ALL) cases [69]. Consistently, inhibition of Wnt signaling by overexpressing the dominant negative form of β-catenin leads to the reduced proliferation of thymocytes. It has been shown that 85 % of the T-ALL patient samples represent elevated levels of β-catenin expression as well as Wnt target genes including c-Myc [70].

At least in two different stages of leukemogenesis, the deregulation of Wnt signaling is playing a role. First of all the initiation phase of the disease, high level of Wnt signaling could be the important factor to progress from a pre-LSC into an LSC (as for AML) and as has been recently investigated for T-ALL where the absence of the Wnt-nuclear factor Tcf1 appears to predispose to T-ALL development (chapter 6). Secondly, in the progression of established disease, as described for CML, ectopically activated Wnt signaling is essential. Thus, depending on the differentiation status of the cell and/or the localization of the leukemia-initiating cells in its niche, Wnt signaling plays various roles during leukemogenesis.

There seem to be at least five different mechanisms playing a role in the deregulation of Wnt signaling during leukemias. First, aberrant levels of Wnt proteins (and/or Wnt antagonists) can be secreted by tumor cells themselves and/or the microenvironment, frequently reported as an autocrine feedback loop of the tumor cells. Second, the sensitivity of the tumor cells towards the Wnt proteins (and antagonists) might be modified (for instance alteration in Fzd, Ror or LRP expression). Thirdly, epigenetic changes are repeatedly observed in most types of leukemias. Both methylation of Wnt antagonists (thereby

interfering with their suppressive function) and of the Wnt5a promoter (leading to low levels of Wnt5a, which acts a tumor suppressor) have been discovered. Fourth, activating mutations in β-catenin or inactivating mutations in APC, or Axin has been described in ALL, and fifth, the balance of the Tcf/Lef factors within a tumor cell appears to be an important determining factor whether Wnt signaling derails during development.

Needless to say, these changes in Wnt signaling offer possibilities for therapeutic intervention, especially because the levels of Wnt signaling in hematological malignancies are significantly higher than their normal counterparts [14, 71]. Moreover, combined with classical abnormalities that are targeted by tyrosine kinase inhibitors in CML (imatinib, neratinib) [72, 73], Notch inhibitors in T-ALL [74], BTK inhibitors in precursor B-ALL (ibrutinib) [75], the dosage needed is expected to be lower as one can envision combination therapy targeting Wnt signaling together with the other genetic alterations to work synergistically. Therefore, the fundamental knowledge about the regulation of normal and malignant development can be rapidly translated into clinical practice.

Future perspectives

In recent years, there has been a serious attempt to expand HSCs by using cytokines or other signals [76, 77]. Among signaling pathways the main focus is denoted into the Wnt and Notch signaling pathways as they are the most evolutionary conserved pathways during hematopoiesis and thymopoiesis [78]. Therefore, there is a great attraction in applying these pathways for ex vivo expansion of HSCs for transplantation purposes [79]. Zon et al. have shown that prostaglandin E2 (PGE2), can induce the expansion of human HSCs via activation of Wnt signaling [80] [81]. The effect of Wnt3a on HSC expansion before transplantation has also been investigated, however, the dosage might have been too high to induce positive effects [10]. It has been shown that the combination of inducing Wnt signaling by using inhibitors of GSK3-β on the one hand and blocking of mTOR pathway by using rapamycin on the other hand [26], results in pro-survival signals in HSCs. Interestingly, activation of canonical Wnt signaling together with the inhibition of mTOR signaling increased the number of murine long-term HSCs in vivo. Under cytokine-free conditions, similar effects were also observed in human and mouse long-term HSCs ex vivo [26].

Our data described in chapters 2-4 indicate that optimal concentration of the activating agents is crucial to provide just the right level of Wnt signaling required for stem cell expansion [32]. In chapter 4 we showed that optimal activation of Wnt signaling in transplanted stem cells could lead to the accelerated T cell reconstitution in vitro and in vivo. In fact, acute myelogenous leukemia (AML) stem cells also require Wnt/β-catenin signaling for their survival [13] though at higher levels than normal HSCs [14]. Therefore, it is essential to understand the correct kinetic and optimal dosages of the Wnt signaling pathway at the different context, cell type and stages of the development.

As Wnt signaling is important for the survival and proliferation of both stem cells and cancer cells, the manipulation of this pathway, either positively or negatively, may be of therapeutic significance. The controlled induction of the Wnt pathway could be beneficial for stem cell repopulation and T cell reconstitution when regeneration is required. On the other hand, the downregulation of Wnt signaling could be used as an effective strategy to prevent uncontrolled self-renewal and expansion of tumorigenic cells.

In chapter 5 we unravel the transcriptional hierarchy driving T cell development in the mouse. The network of transcription factors plays two crucial roles at the early stages of T cell development; Firstly proliferation of T cell progenitors in order to establish an adequate pool of cells, secondly the inhibition of expansion and upregulation of T specific genes and TCR signaling. Precise tuning of these two stages becomes possible via the sophisticated transcriptional network and is required for a successful T cell commitment. While some of

these factors should be highly expressed in phase 1, the same factor should be irreversibly silent for the initiation and completion of phase 2. The results obtained in chapter 5 should also be examined in human stem cells and thymocyte differentiation schemes. This is important, as, despite many fundamental similarities, significant differences between mice and humans exist in the stem cell compartment and thymus alike, necessitating the study of the human immune system not only to understand our own species but also to translate findings into clinical treatment modalities. As the thymus is not only involved in rare diseases such as SCID and DiGeorge syndrome, but also target of more common viral infections (e.g., HIV disease), directly involved in auto immune diseases, and involuted during ageing, leading to senescent T cell responses, better knowledge of human T cell development (often using concepts first discovered in the mouse system) is crucial in a wide variety of diseases and conditions.

On the way to translate fundamental knowledge into clinical settings several in vitro and in vivo models have been developed to closely mimic the human physiological situations. One of the interesting models being used recently is humanized mouse models e.g. NSG and NOG [82-83]. Combing advanced genetic techniques such as the CRISPR-CAS9 system, which allow the loss of function studies on human cells, with the NSG xenotransplantation models would shed important light on the biology of human hematopoiesis and thymopoiesis.

Another intriguing in vitro model is using induced pluripotent stem cells (iPSC) derived HSCs and T cells. Establishment of a robust protocol for differentiation of iPSC's towards HSCs and T cells would provide an outstanding opportunity for the translation of our basic knowledge obtained from the mouse into a clinically relevant model. The model could not only be applied in regenerative medicine but also could be used in drug discovery via high throughput efficacy screening in various iPSC derived disease models. A fascinating HTS platform is the library of Wnt signaling modulators which might result in the discovery of new chemical entities (NCEs) with the ability to better control the right dosage of Wnt signaling in order to accelerate T cell reconstitution in healthy conditions or to inhibit the growth of cancer stem cells in hematological malignancies.

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Summary in English

The immune system of mammals is responsible for protecting our body against pathogens and foreign substances (antigens), and it consists of two discrete lines of defense. The first line called innate immunity and provide a quick and nonspecific defense. The innate immunity includes different cells types, such as mast cells, macrophages, neutrophils, eosinophils, dendritic cells and natural killer (NK) cells. The second line of defense called adaptive immunity responds in an antigen-specific manner, and comprised of B and T lymphocyte cells.

All types of immune cells mentioned above are derived from a unique cell type that resides in bone marrow (BM), called hematopoietic stem cells (HSCs). This rare cell type is characterized as multipotent cells because they can differentiate into any type of immune cells just by receiving the "right" signal, but also they have the potential to repopulate (self-renewal) in order to maintain enough pool of precursor cells.

All classes of immune cells except T lymphocytes develop and maturate in the bone marrow. For T cell development, HSCs should migrate into the thymus via the circulation and seed the thymus. Thymic seeding progenitors (formerly HSCs) will first expand in the thymus in order to sustain sufficient pool of progenitor T cells and subsequently they develop towards mature and functional T cells. It is known that thymus is the only organ in our body which could provide right signals for T cell development.

This thesis focuses on one of the signals which are known to play an important role during HSC repopulation and T cell development that is Wnt signaling pathway. Depending on the tissue/cell types (microenvironment) and specific class of Wnt proteins binding to the corresponding receptors on the developing lymphocytes, two discrete downstream pathways will be activated namely canonical or non-canonical Wnt pathway. The main aim of this thesis is to dissect the roles of these two distinct pathways during hematopoiesis and lymphocyte development in murine as a physiologically relevant animal model.

In **chapter 2** we investigated the role of canonical Wnt signaling in HSCs repopulation and differentiation in the BM. One of the most common approaches to study roles of genes is manipulation of the genes of interest e.g. knocking out or overexpressing by mutations. In this chapter, we used such a genetic tool to overexpress canonical Wnt signaling at various levels (low to high) in HSCs. The aim of this study is to solve the controversy of the previous studies observed due to the application of different gain of function and loss of function genetic models. Our results show that high levels of Wnt signaling results in the loss of stem cell repopulation. We further explored the mechanisms underlying this phenomenon using gene expression and functional analysis approaches. Our data revealed that high

levels of Wnt signaling in HSCs is in favor of differentiation thereby reduce the self-renewal of stem cells. This led to the exhaustion of HSCs pool. Therefore, an optimal dosage of Wnt signaling is crucial for the homeostasis of HSCs by maintaining sufficient pool on one hand and the induction of differentiation towards mature immune cells on the other hand.

In **chapter 3** we focused on the role of non-canonical Wnt signaling in HSCs by using another genetic model in which one of the non-canonical Wnt receptors called Ryk is knocked out. We performed functional experiments by transplanting the Ryk KO stem cells into the irradiated mice and monitored the reconstitution of the immune system over time. Our data suggest that the absence of non-canonical Wnt signaling via Ryk deficiency results in a mild decrease of stem cell repopulation. Further mechanistic studies showed that the loss of stemness is caused by an increased apoptosis (programmed cell death) and decreased proliferation in the HSCs

In **chapter 4** we performed a side by side study of the effect canonical and non-canonical Wnt signaling in lymphocyte and in particular T cell development. Here we used a gain of function approach by overexpressing canonical Wnt ligands (Wnt3a) and non-canonical Wnt ligand (Wnt5a). Our in vitro studies revealed that high levels of Wnt signaling inhibit T cell development while intermediate levels accelerate this process, confirming that the optimal dosage of Wnt signaling is also crucial during T cell development in the thymus. On the other hand, activation of Wnt5a non-canonical Wnt signaling is harmful to the T lymphopoiesis and increase apoptosis in the developing T cell progenitors. Our in vivo studies (transplantation assays) showed that overexpression of Wnt3a is in favor of lymphopoiesis while overexpression of non-canonical pathways promotes myeloid differentiation. The latter considered as an inefficient hematopoiesis which happens at the older ages and known as senescence of the stem cells. These findings (**chapters 2-4**) enhanced our understanding of the biology of HSCs and T cell development and could help us to develop more efficient protocols for HSC expansion and T cell reconstitution in future.

Aberrant Wnt signaling, e.g. genetic mutations in one of the key components of the pathway, has been reported in various types of leukemia and lymphomas (white blood cell's cancer). Mutations in TCF-1 (T cell factor) has been shown in several patients with leukemia. TCF-1 is a crucial transcription factor during T cell development and its expression is regulated by canonical Wnt signaling pathway. In **chapters 5 and 6** we studied the role Tcf-1 during normal T cell development (**chapter 5**) and during an occurrence of thymic malignancy (**chapter 6**) by using a loss of function model of TCF-1.

Our data in **chapter 5** revealed that deficiency in Tcf-1 results in partial blocks at various stages of T cell development while inducing development of non-T cells within the

thymus. Overexpression of Bcl-11b (another crucial gene for T cell commitment) rescues the T cell development even in the absence of Tcf-1 showing that Tcf-1 functions via Bcl-11b to promote T cell development. However, in order to suppress the development of non-T cells, we upregulated another gene called Gata-3. These results reveal that T cell development is controlled by a minimal transcription factor network involving Tcf1, and the subsequent division of labor between Bcl11b and Gata3, thereby ensuring a properly regulated T cell gene expression program.

At older ages, mice with deficiency of Tcf-1 develop highly metastatic thymic lymphoma. In **chapter 6** we performed mechanistic studies to understand the cause of lymphoma development. Deregulation of Wnt signaling (high expression of Wnt target genes) observed in the leukemic T cells. Further studies revealed that Tcf1 is higher expressed than Lef1 (another transcription factor downstream of Wnt pathway), with a predominance of Wnt inhibitory isoforms. Loss of Tcf1 as the repressor of Lef1 leads to high Wnt activity and is the initiating event in lymphoma development. Thus, we showed that Tcf1 acts as a molecular switch between proliferative and repressive signals during T-lymphocyte development in the thymus.

It has been a long-standing challenge to mimic the physiological signals in vitro or ex vivo, with the aim to proliferate and manipulate HSCs and/or thymocytes for clinical purposes. The possibility of stimulating these cells with Wnt ligands is a very attractive approach since this would result in a more controlled therapy, compared to approaches requiring permanent gene modification. This would ultimately result in a faster thymic reconstitution by thymic seeding cells. The finding obtained in this thesis could be used for the above-mentioned purposes in future.

Nederlandse samenvatting

Het afweersysteem van zoogdieren is verantwoordelijk voor de bescherming van ons lichaam tegen ziekteverwekkers en lichaamsvreemde stoffen (antigenen), en het bestaat uit twee afzonderlijke verdedigingslinies. De eerste lijn, de aangeboren immuniteit zorgt voor een snelle en niet-specifieke verdediging. Deze aangeboren immuniteit omvat verschillende soorten cellen, zoals mestcellen, macrofagen, neutrofielen, eosinofielen, dendritische cellen en natural killercellen (NK). De tweede verdedigingslinie de adaptieve immuniteit, reageert op een antigen-specifieke manier en bestaat uit witte bloedcellen (ook bekend als B- en T-lymfocytcellen).

Alle typen immuun cellen die hierboven zijn genoemd, zijn afkomstig van een uniek celtype dat in het beenmerg (BM) voorkomt, de zogenaamde hematopoietische stamcel (HSC). Dit zeldzame celtype wordt gekarakteriseerd als multipotent omdat ze kunnen differentiëren in elk type immuun cellen wanneer ze het "juiste" signaal te ontvangen, maar ook omdat ze beschikken over zelfvernieuwing om daarmee een voldoende grote pool van precursorcellen te behouden.

Alle typen immuun cellen behalve T-lymfocyten ontwikkelen zich in het beenmerg. Voor de ontwikkeling van T-cellen moeten HSC's migreren naar de thymus via de bloedsomloop en de thymus bevolken. Thymic-seeding-voorlopers zullen eerst expanderen in de thymus om een groot genoeg pool van voorlopercellen van T-cellen in stand te houden en vervolgens ontwikkelen ze zich tot rijpe en functionele T-cellen. Het is bekend dat thymus het enige orgaan in ons lichaam is dat de juiste signalen kan geven voor de ontwikkeling van T-cellen.

Dit proefschrift richt zich op een van de signalen waarvan bekend is dat ze een belangrijke rol spelen tijdens HSC-repopulatie en ontwikkeling van T-cellen, nl. de zogenaamde die Wnt-signaalroute. Afhankelijk van het weefsel of celtype (micro-omgeving) en van de specifieke klasse van Wnt-eiwitten die binden aan hun specifieke receptoren op de zich ontwikkelende lymfocyten, zullen twee verschillende signaalroutes worden geactiveerd, namelijk de canonieke of niet-canonieke Wnt-route. Het hoofddoel van dit proefschrift is om de rollen van deze twee verschillende routes te ontleden tijdens de ontwikkeling van bloed stamcellen en lymfocyten. Dit wordt gedaan in de muis als fysiologisch relevant diermodel.

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Now that I get to write the acknowledgment of my thesis I start believing that this long journey is coming to an end. Although it is still hard to believe!!

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Curriculum Vitae

Farbod Famili was born on June 1st, 1985 in Isfahan, Iran. He studied Bachelor of Biology in the Faculty of science at Isfahan University, Isfahan, Iran in 2008. After obtaining his diploma, he moved to the Netherlands and started a Master program in the field of Biotechnology. He obtained MSc of Medical Biotechnology from Wageningen University, Wageningen, the Netherlands in 2010. During his studies, Farbod performed an internship at the Department of Gastroenterology and Hepatology at Erasmus Medical Center in Rotterdam, the Netherlands. His research was focused on the role of human NK cells in liver transplantation.

Farbod started his PhD studies in the Department of ImmunoHematology and Blood transfusion in Leiden University Medical Center, Leiden, the Netherlands in 2010. The work during his PhD period is subject of this thesis. Farbod finalized practical part of his studies in 2015 and subsequently, he joined Charles River Laboratories in Leiden, the Netherlands, to function as an assay development scientist in the field of lung fibrosis. Currently, Farbod is employed at Ncardia.B.V, Leiden, the Netherlands, where he functions as a senior scientist of drug discovery and development to develop high-throughput compatible assays using the proprietary model of hIPSC-derived cardiomyocytes.

List of Publications

Tcf1 regulates T lymphocyte lineage fidelity through its target genes Gata3 and Bcl11b. Farbod Famili, Laura Garcia Perez, Marja van Eggermond, Haoyu Wu, Martijn Brugman, Martijn Cordes, Machteld M. Tiemessen, Karin Pike-Overzet, Lucia Clemems-Daxinger, Frank J.T. Staal. Manuscript submitted

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