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

Famili, F.

CHAPTER 1

General Introduction

Parts of this chapter were published in:
The development of T cells from stem cells in mice and humans.
Future Sci. OA (2017) FSO186
Aberrant Wnt Signaling in Leukemia.
Cancers 2016, 8, 78

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].

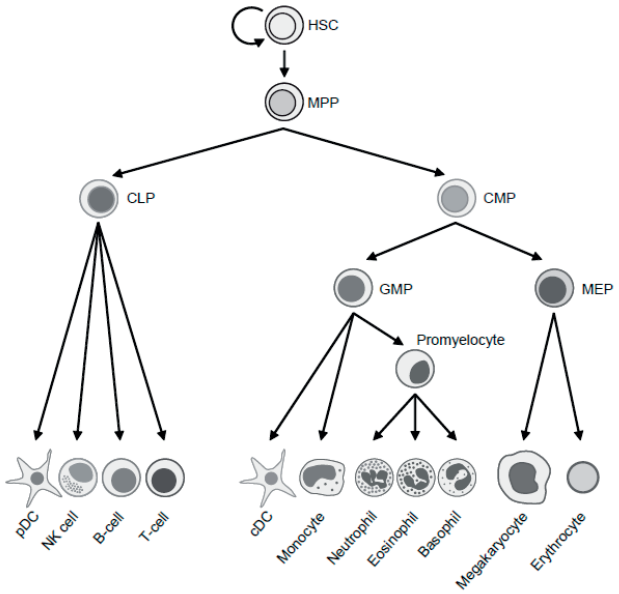

Figure 1. Schematic representation of the main lineage commitment steps in hematopoiesis.

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].

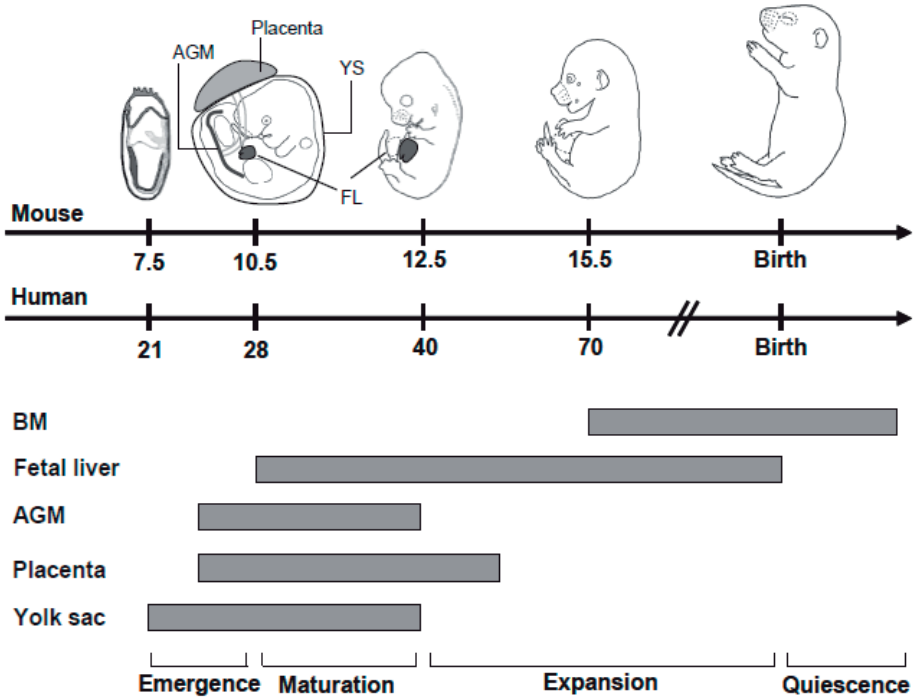

Figure 2. Main anatomical sites of hematopoiesis during embryonic development.

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.

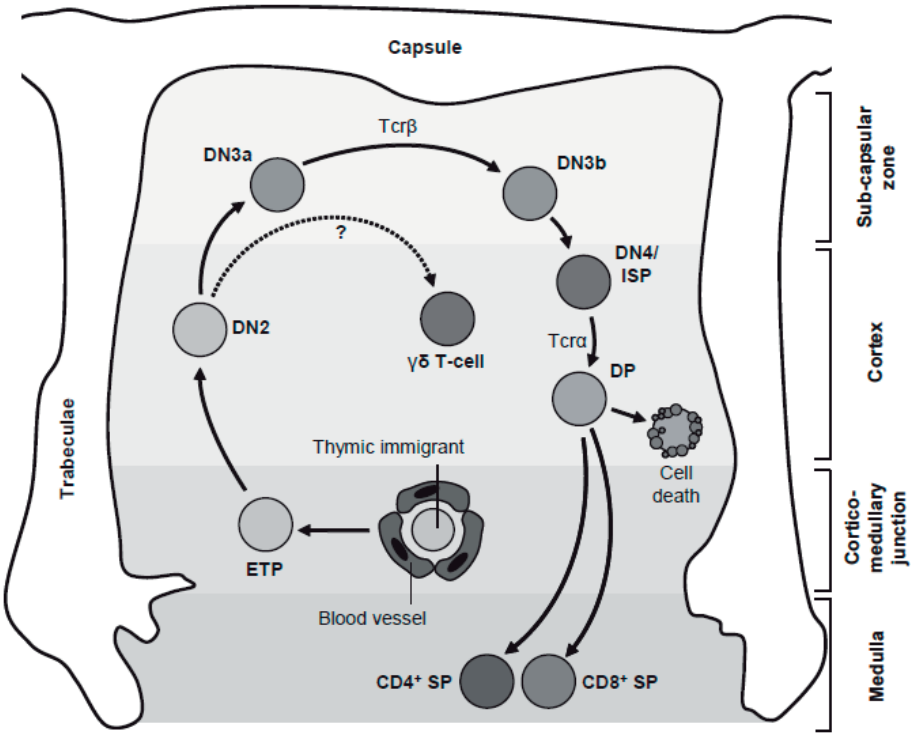

Figure 3. T cell developmental stages in the thymus.

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

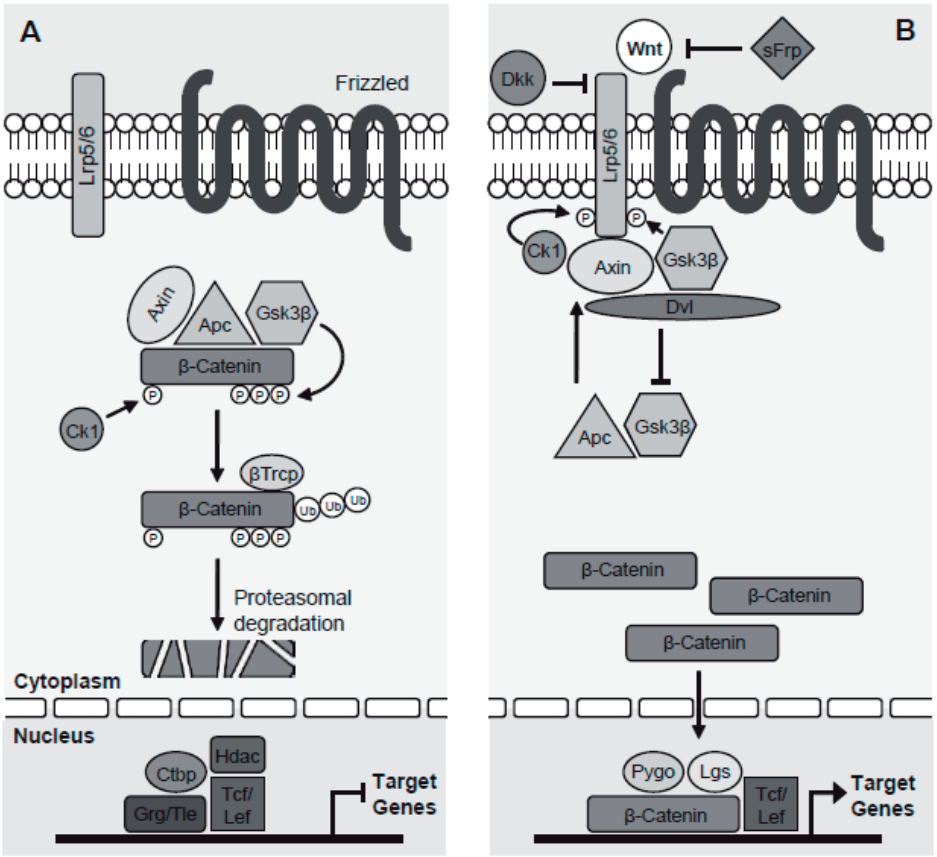

Figure 4. Canonical or Wnt-β-catenin-Tcf/Lef signaling.

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.

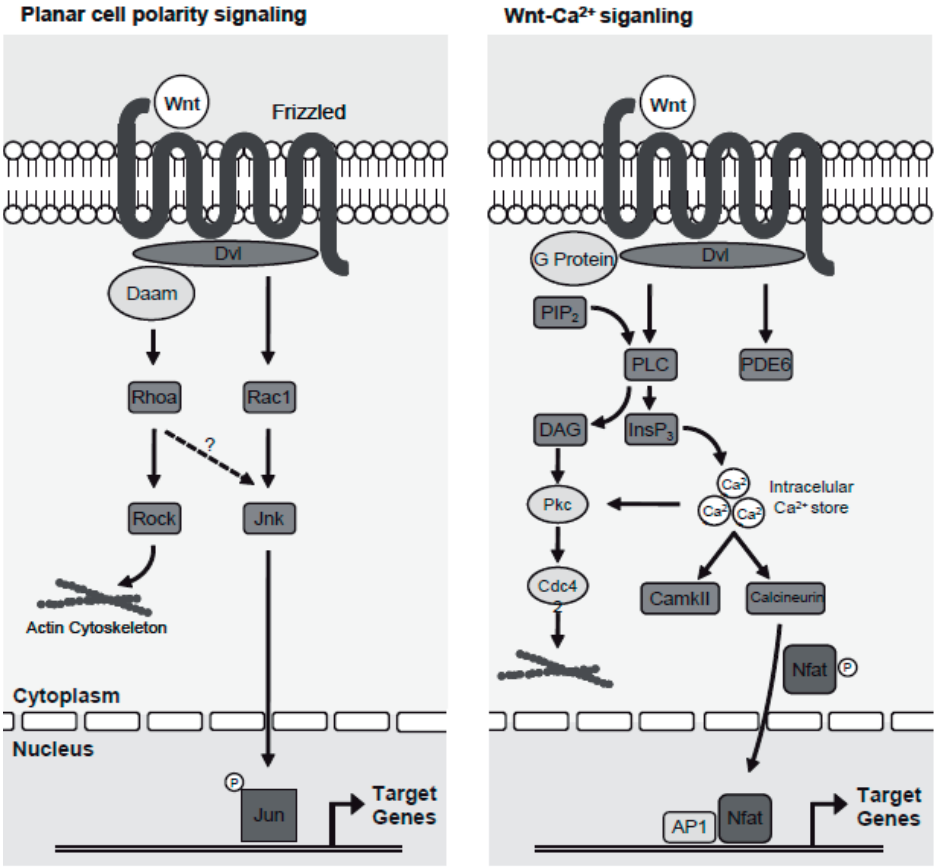

Figure 5. Non-canonical Wnt signaling.

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].

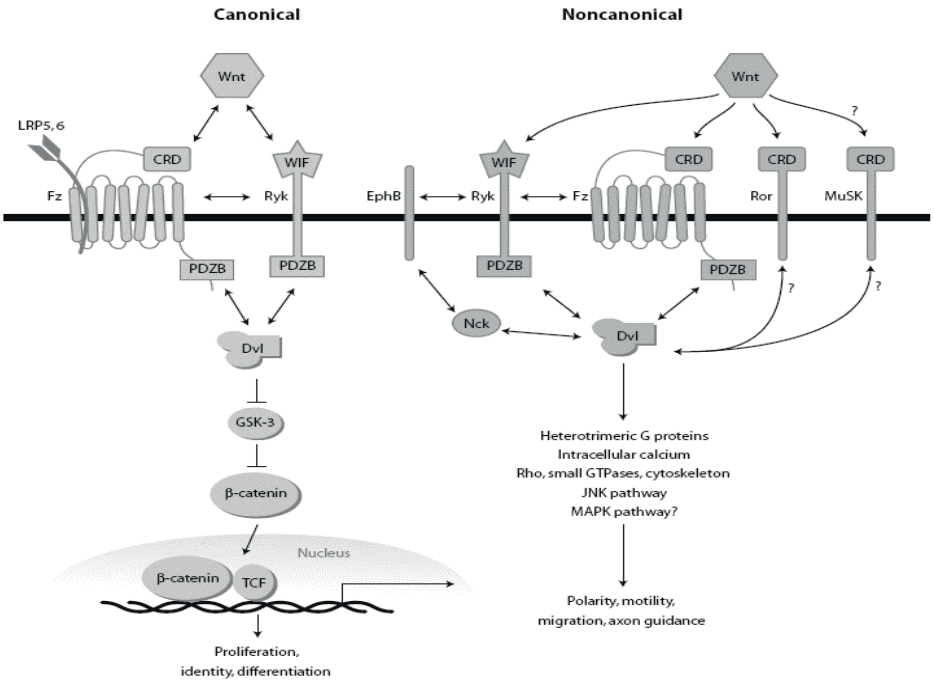

Figure 6. Cooperative binding of Ryk to Wnt, Dvl, or adaptor and scaffold proteins.

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.

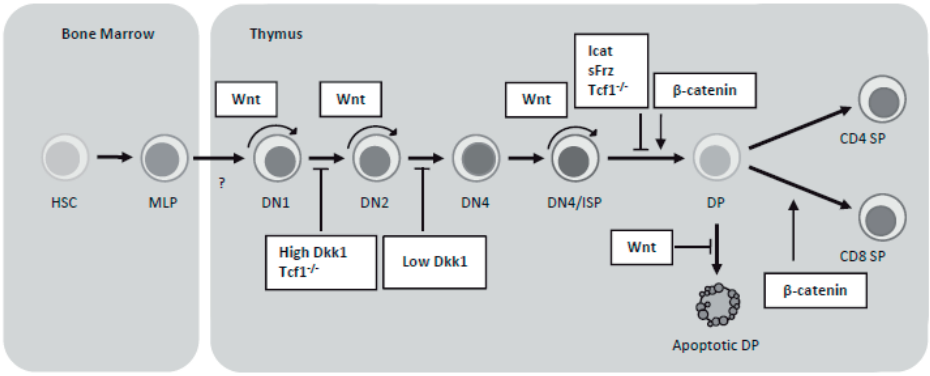

Figure 7. Effect of Wnt signaling during thymopoiesis in the thymus.

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.

References

1. Milner, L.A. and A. Bigas, Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood, 1999. 93(8): p. 2431-48.
2. Luis, T.C., et al., Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. Leukemia, 2012. 26(3): p. 414-21.
3. Singbrant, S., et al., Canonical BMP signaling is dispensable for hematopoietic stem cell function in both adult and fetal liver hematopoiesis, but essential to preserve colon architecture. Blood, 2010. 115(23): p. 4689-98.
4. Blank, U., G. Karlsson, and S. Karlsson, Signaling pathways governing stem-cell fate. Blood, 2008. 111(2): p. 492-503.
5. Dzierzak, E. and N.A. Speck, Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol, 2008. 9(2): p. 129-36.
6. Eaves, C.J., Hematopoietic stem cells: concepts, definitions, and the new reality. Blood, 2015. 125(17): p. 2605-13.
7. Fuchs, E. and J.A. Segre, Stem cells: a new lease on life. Cell, 2000. 100(1): p. 143-55.
8. Spangrude, G.J., S. Heimfeld, and I.L. Weissman, Purification and characterization of mouse hematopoietic stem cells. Science, 1988. 241(4861): p. 58-62.
9. Morrison, S.J., et al., The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci U S A, 1995. 92(22): p. 10302-6.
10. Staal, F.J., et al., The functional relationship between hematopoietic stem cells and developing T lymphocytes. Ann N Y Acad Sci, 2016. 1370(1): p. 36-44.
11. Smith, L.G., I.L. Weissman, and S. Heimfeld, Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc Natl Acad Sci U S A, 1991. 88(7): p. 2788-92.
12. Morrison, S.J. and I.L. Weissman, The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity, 1994. 1(8): p. 661-73.
13. Osawa, M., et al., Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science, 1996. 273(5272): p. 242-5.
14. Uchida, N. and I.L. Weissman, Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin-Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med, 1992. 175(1): p. 175-84.
15. Kim, I., et al., Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. Blood, 2006. 108(2): p. 737-44.
16. Sintes, J., et al., Differential expression of CD150 (SLAM) family receptors by human hematopoietic stem and progenitor cells. Exp Hematol, 2008. 36(9): p. 1199-204.
17. Grunebaum, E., et al., Bone marrow transplantation for severe combined immune deficiency. JAMA, 2006. 295(5): p. 508-18.
18. Ng, Y.Y., et al., Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol, 2004. 75(2): p. 314-23.
19. Bhatia, M., et al., Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med, 1997. 186(4): p. 619-24.
20. Majeti, R., et al., Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. Proc Natl Acad Sci U S A, 2009. 106(9): p. 3396-401.
21. Notta, F., et al., Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science, 2011. 333(6039): p. 218-21.

22. van Galen, P., et al., Reduced lymphoid lineage priming promotes human hematopoietic stem cell expansion. Cell Stem Cell, 2014. 14(1): p. 94-106.
23. Notta, F., et al., Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science, 2016. 351(6269): p. aab2116.
24. Allman, D., et al., Thymopoiesis independent of common lymphoid progenitors. Nat Immunol, 2003. 4(2): p. 168-74.
25. Kondo, M., I.L. Weissman, and K. Akashi, Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell, 1997. 91(5): p. 661-72.
26. Sitnicka, E., et al., Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. Immunity, 2002. 17(4): p. 463-72.
27. Mansson, R., et al., Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity, 2007. 26(4): p. 407-19.
28. Spits, H., et al., Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol, 2013. 13(2): p. 145-9.
29. Spits, H., J.H. Bernink, and L. Lanier, NK cells and type 1 innate lymphoid cells: partners in host defense. Nat Immunol, 2016. 17(7): p. 758-64.
30. Spits, H. and J.P. Di Santo, The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat Immunol, 2011. 12(1): p. 21-7.
31. Laurenti, E., et al., The transcriptional architecture of early human hematopoiesis identifies multi-level control of lymphoid commitment. Nat Immunol, 2013. 14(7): p. 756-63.
32. Orkin, S.H. and L.I. Zon, Hematopoiesis: an evolving paradigm for stem cell biology. Cell, 2008. 132(4): p. 631-44.
33. Martins, V.C., et al., Cell competition is a tumour suppressor mechanism in the thymus. Nature, 2014. 509(7501): p. 465-70.
34. Brummendorf, T.H., et al., Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. J Exp Med, 1998. 188(6): p. 1117-24.
35. Takano, H., et al., Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med, 2004. 199(3): p. 295-302.
36. Wu, M., et al., Imaging hematopoietic precursor division in real time. Cell Stem Cell, 2007. 1(5): p. 541-54.
37. Foudi, A., et al., Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nat Biotechnol, 2009. 27(1): p. 84-90.
38. Wilson, A., et al., Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell, 2008. 135(6): p. 1118-29.
39. Wilson, A., E. Laurenti, and A. Trumpp, Balancing dormant and self-renewing hematopoietic stem cells. Curr Opin Genet Dev, 2009. 19(5): p. 461-8.
40. Cumano, A. and I. Godin, Ontogeny of the hematopoietic system. Annu Rev Immunol, 2007. 25: p. 745-85.
41. Ferkowicz, M.J., et al., CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. Development, 2003. 130(18): p. 4393-403.
42. Palis, J., et al., Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development, 1999. 126(22): p. 5073-84.
43. Cumano, A., F. Dieterlen-Lievre, and I. Godin, Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell, 1996. 86(6): p. 907-16.

44. Medvinsky, A. and E. Dzierzak, Definitive hematopoiesis is autonomously initiated by the AGM region. Cell, 1996. 86(6): p. 897-906.
45. de Bruijn, M.F., et al., Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity, 2002. 16(5): p. 673-83.
46. Bertrand, J.Y., et al., Haematopoietic stem cells derive directly from aortic endothelium during development. Nature, 2010. 464(7285): p. 108-11.
47. Boisset, J.C., et al., In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature, 2010. 464(7285): p. 116-20.
48. Jaffredo, T., et al., Tracing the progeny of the aortic hemangioblast in the avian embryo. Dev Biol, 2000. 224(2): p. 204-14.
49. Kissa, K. and P. Herbomel, Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature, 2010. 464(7285): p. 112-5.
50. Bertrand, J.Y., et al., Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. Proc Natl Acad Sci U S A, 2005. 102(1): p. 134-9.
51. Kumaravelu, P., et al., Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development, 2002. 129(21): p. 4891-9.
52. Houssaint, E., Differentiation of the mouse hepatic primordium. II. Extrinsic origin of the haemopoietic cell line. Cell Differ, 1981. 10(5): p. 243-52.
53. Johnson, G.R. and M.A. Moore, Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. Nature, 1975. 258(5537): p. 726-8.
54. Dzierzak, E., The emergence of definitive hematopoietic stem cells in the mammal. Curr Opin Hematol, 2005. 12(3): p. 197-202.
55. Mikkola, H.K. and S.H. Orkin, The journey of developing hematopoietic stem cells. Development, 2006. 133(19): p. 3733-44.
56. Ema, H. and H. Nakauchi, Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. Blood, 2000. 95(7): p. 2284-8.
57. Rebel, V.I., et al., The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. Blood, 1996. 87(8): p. 3500-7.
58. Wilson, A. and A. Trumpp, Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol, 2006. 6(2): p. 93-106.
59. Aspinall, R. and D. Andrew, Thymic involution in aging. J Clin Immunol, 2000. 20(4): p. 250-6.
60. Steinmann, G.G., B. Klaus, and H.K. Muller-Hermelink, The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. Scand J Immunol, 1985. 22(5): p. 563-75.
61. Manley, N.R., Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. Semin Immunol, 2000. 12(5): p. 421-8.
62. Shinohara, T. and T. Honjo, Studies in vitro on the mechanism of the epithelial/mesenchymal interaction in the early fetal thymus. Eur J Immunol, 1997. 27(2): p. 522-9.
63. van Ewijk, W., E.W. Shores, and A. Singer, Crosstalk in the mouse thymus. Immunol Today, 1994. 15(5): p. 214-7.
64. Boyd, R.L., et al., The thymic microenvironment. Immunol Today, 1993. 14(9): p. 445-59.
65. Rothenberg, E.V., J.E. Moore, and M.A. Yui, Launching the T-cell-lineage developmental programme. Nat Rev Immunol, 2008. 8(1): p. 9-21.
66. Peaudecerf, L., et al., Thymocytes may persist and differentiate without any input from bone marrow progenitors. J Exp Med, 2012. 209(8): p. 1401-8.

67. Dik, W.A., et al., New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med, 2005. 201(11): p. 1715-23.
68. Scollay, R., P. Bartlett, and K. Shortman, T cell development in the adult murine thymus: changes in the expression of the surface antigens Ly2, L3T4 and B2A2 during development from early precursor cells to emigrants. Immunol Rev, 1984. 82: p. 79-103.
69. Weerkamp, F., et al., Human thymus contains multipotent progenitors with T/B lymphoid, myeloid, and erythroid lineage potential. Blood, 2006. 107(8): p. 3131-7.
70. Lind, E.F., et al., Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. J Exp Med, 2001. 194(2): p. 127-34.
71. Bhandoola, A. and A. Sambandam, From stem cell to T cell: one route or many? Nat Rev Immunol, 2006. 6(2): p. 117-26.
72. Shortman, K. and L. Wu, Early T lymphocyte progenitors. Annu Rev Immunol, 1996. 14: p. 29-47.
73. Arinobu, Y., et al., Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. Cell Stem Cell, 2007. 1(4): p. 416-27.
74. Yoshida, T., et al., Early hematopoietic lineage restrictions directed by Ikaros. Nat Immunol, 2006. 7(4): p. 382-91.
75. Adolfsson, J., et al., Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell, 2005. 121(2): p. 295-306.
76. Homminga, I., et al., Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. Cancer Cell, 2011. 19(4): p. 484-97.
77. Matsuzaki, Y., et al., Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. J Exp Med, 1993. 178(4): p. 1283-92.
78. Porritt, H.E., et al., Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity, 2004. 20(6): p. 735-45.
79. Wu, L., et al., Developmental potential of the earliest precursor cells from the adult mouse thymus. J Exp Med, 1991. 174(6): p. 1617-27.
80. Hozumi, K., et al., Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med, 2008. 205(11): p. 2507-13.
81. Jaleco, A.C., et al., Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. J Exp Med, 2001. 194(7): p. 991-1002.
82. Koch, U., et al., Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med, 2008. 205(11): p. 2515-23.
83. Pui, J.C., et al., Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity, 1999. 11(3): p. 299-308.
84. Radtke, F., et al., Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity, 1999. 10(5): p. 547-58.
85. Ciofani, M. and J.C. Zuniga-Pflucker, Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. Nat Immunol, 2005. 6(9): p. 881-8.
86. Stier, S., et al., Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood, 2002. 99(7): p. 2369-78.
87. Varnum-Finney, B., C. Brashem-Stein, and I.D. Bernstein, Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. Blood, 2003. 101(5): p. 1784-9.
88. Staal, F.J. and H.C. Clevers, Wnt signaling in the thymus. Curr Opin Immunol, 2003. 15(2): p. 204-8.

89. Staal, F.J. and H.C. Clevers, WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol, 2005. 5(1): p. 21-30.
90. Petrie, H.T. and J.C. Zuniga-Pflucker, Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol, 2007. 25: p. 649-79.
91. van Dongen, J.J., et al., Development of human T lymphocytes and their thymus-dependency. Thymus, 1990. 16(3-4): p. 207-34.
92. Yui, M.A., N. Feng, and E.V. Rothenberg, Fine-scale staging of T cell lineage commitment in adult mouse thymus. J Immunol, 2010. 185(1): p. 284-93.
93. Taghon, T., et al., Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity, 2006. 24(1): p. 53-64.
94. Capone, M., R.D. Hockett, Jr., and A. Zlotnik, Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. Proc Natl Acad Sci U S A, 1998. 95(21): p. 12522-7.
95. Staal, F.J., T.C. Luis, and M.M. Tiemessen, WNT signalling in the immune system: WNT is spreading its wings. Nat Rev Immunol, 2008. 8(8): p. 581-93.
96. von Boehmer, H., Aspects of lymphocyte developmental biology. Immunol Today, 1997. 18(6): p. 260-2.
97. Kisielow, P. and H. von Boehmer, Development and selection of T cells: facts and puzzles. Adv Immunol, 1995. 58: p. 87-209.
98. Surh, C.D. and J. Sprent, T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature, 1994. 372(6501): p. 100-3.
99. Nusse, R., et al., A new nomenclature for int-1 and related genes: the Wnt gene family. Cell, 1991. 64(2): p. 231.
100. Nusse, R., et al., Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature, 1984. 307(5947): p. 131-6.
101. Brembeck, F.H., M. Rosario, and W. Birchmeier, Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr Opin Genet Dev, 2006. 16(1): p. 51-9.
102. Clevers, H., Wnt/beta-catenin signaling in development and disease. Cell, 2006. 127(3): p. 469-80.
103. Aberle, H., et al., beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J, 1997. 16(13): p. 3797-804.
104. Staal, F.J., et al., Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep, 2002. 3(1): p. 63-8.
105. Roose, J., et al., The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature, 1998. 395(6702): p. 608-12.
106. Davidson, G., et al., Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. Nature, 2005. 438(7069): p. 867-72.
107. Staal, F.J., et al., Wnt target genes identified by DNA microarrays in immature CD34+ thymocytes regulate proliferation and cell adhesion. J Immunol, 2004. 172(2): p. 1099-108.
108. Zeng, X., et al., A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature, 2005. 438(7069): p. 873-7.
109. Kawano, Y. and R. Kypta, Secreted antagonists of the Wnt signalling pathway. J Cell Sci, 2003. 116(Pt 13): p. 2627-34.
110. Hsieh, J.C., et al., A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature, 1999. 398(6726): p. 431-6.
111. Mao, B., et al., Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature, 2002. 417(6889): p. 664-7.

112. Schuijers, J. and H. Clevers, Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J, 2012. 31(12): p. 2685-96.
113. Jin, Y.R. and J.K. Yoon, The R-spondin family of proteins: emerging regulators of WNT signaling. Int J Biochem Cell Biol, 2012. 44(12): p. 2278-87.
114. de Lau, W., et al., The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. Genes Dev, 2014. 28(4): p. 305-16.
115. Daniels, D.L. and W.I. Weis, ICAT inhibits beta-catenin binding to Tcf/Lef-family transcription factors and the general coactivator p300 using independent structural modules. Mol Cell, 2002. 10(3): p. 573-84.
116. van de Wetering, M., et al., The human T cell transcription factor-1 gene. Structure, localization, and promoter characterization. J Biol Chem, 1992. 267(12): p. 8530-6.
117. Kuwahara, A., et al., Wnt signaling and its downstream target N-myc regulate basal progenitors in the developing neocortex. Development, 2010. 137(7): p. 1035-44.
118. Lustig, B., et al., Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol, 2002. 22(4): p. 1184-93.
119. Tetsu, O. and F. McCormick, Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature, 1999. 398(6726): p. 422-6.
120. Wielenga, V.J., et al., Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol, 1999. 154(2): p. 515-23.
121. Liang, H., et al., Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. Cancer Cell, 2003. 4(5): p. 349-60.
122. Liang, H., et al., Noncanonical Wnt signaling promotes apoptosis in thymocyte development. J Exp Med, 2007. 204(13): p. 3077-84.
123. Semenov, M.V., et al., SnapShot: Noncanonical Wnt Signaling Pathways. Cell, 2007. 131(7): p. 1378.
124. Endo, Y., et al., Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2. J Biol Chem, 2005. 280(1): p. 777-86.
125. Kuhl, M., et al., The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet, 2000. 16(7): p. 279-83.
126. Sheldahl, L.C., et al., Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. J Cell Biol, 2003. 161(4): p. 769-77.
127. Clipstone, N.A. and G.R. Crabtree, Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature, 1992. 357(6380): p. 695-7.
128. Graef, I.A., F. Chen, and G.R. Crabtree, NFAT signaling in vertebrate development. Curr Opin Genet Dev, 2001. 11(5): p. 505-12.
129. Murphy, L.L. and C.C. Hughes, Endothelial cells stimulate T cell NFAT nuclear translocation in the presence of cyclosporin A: involvement of the wnt/glycogen synthase kinase-3 beta pathway. J Immunol, 2002. 169(7): p. 3717-25.
130. Dijksterhuis, J.P., J. Petersen, and G. Schulte, WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3. Br J Pharmacol, 2014. 171(5): p. 1195-209.
131. van Amerongen, R., A. Mikels, and R. Nusse, Alternative wnt signaling is initiated by distinct receptors. Sci Signal, 2008. 1(35): p. re9.
132. Hovens, C.M., et al., RYK, a receptor tyrosine kinase-related molecule with unusual kinase domain motifs. Proc Natl Acad Sci U S A, 1992. 89(24): p. 11818-22.
133. Callahan, C.A., et al., Control of neuronal pathway selection by a Drosophila receptor protein-tyrosine kinase family member. Nature, 1995. 376(6536): p. 171-4.

134. Yoshikawa, S., et al., Wnt-mediated axon guidance via the Drosophila Derailed receptor. Nature, 2003. 422(6932): p. 583-8.
135. Fradkin, L.G., J.M. Dura, and J.N. Noordermeer, Ryks: new partners for Wnts in the developing and regenerating nervous system. Trends Neurosci, 2010. 33(2): p. 84-92.
136. Lu, W., et al., Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell, 2004. 119(1): p. 97-108.
137. Simoneaux, D.K., et al., The receptor tyrosine kinase-related gene (ryk) demonstrates lineage and stage-specific expression in hematopoietic cells. J Immunol, 1995. 154(3): p. 1157-66.
138. Povinelli, B.J. and M.J. Nemeth, Wnt5a regulates hematopoietic stem cell proliferation and repopulation through the Ryk receptor. Stem Cells, 2014. 32(1): p. 105-15.
139. Austin, T.W., et al., A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood, 1997. 89(10): p. 3624-35.
140. Van Den Berg, D.J., et al., Role of members of the Wnt gene family in human hematopoiesis. Blood, 1998. 92(9): p. 3189-202.
141. Rattis, F.M., C. Voermans, and T. Reya, Wnt signaling in the stem cell niche. Curr Opin Hematol, 2004. 11(2): p. 88-94.
142. Reya, T., et al., A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature, 2003. 423(6938): p. 409-14.
143. Willert, K., et al., Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature, 2003. 423(6938): p. 448-52.
144. Baba, Y., K.P. Garrett, and P.W. Kincade, Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. Immunity, 2005. 23(6): p. 599-609.
145. Luis, T.C., et al., Wnt3a nonredundantly controls hematopoietic stem cell function and its deficiency results in complete absence of canonical Wnt signaling. Blood, 2010. 116(3): p. 496-7.
146. Luis, T.C., et al., Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell Stem Cell, 2011. 9(4): p. 345-56.
147. Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake, Notch signaling: cell fate control and signal integration in development. Science, 1999. 284(5415): p. 770-6.
148. Radtke, F., et al., Notch regulation of lymphocyte development and function. Nat Immunol, 2004. 5(3): p. 247-53.
149. Staal, F.J., et al., Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. Eur J Immunol, 2001. 31(1): p. 285-93.
150. Mulroy, T., et al., Wnt-1 and Wnt-4 regulate thymic cellularity. Eur J Immunol, 2002. 32(4): p. 967-71.
151. Okamura, R.M., et al., Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. Immunity, 1998. 8(1): p. 11-20.
152. Verbeek, S., et al., An HMG-box-containing T-cell factor required for thymocyte differentiation. Nature, 1995. 374(6517): p. 70-4.
153. Behrens, J., et al., Functional interaction of beta-catenin with the transcription factor LEF-1. Nature, 1996. 382(6592): p. 638-42.
154. Huber, O., et al., Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech Dev, 1996. 59(1): p. 3-10.
155. Schilham, M.W., et al., Critical involvement of Tcf-1 in expansion of thymocytes. J Immunol, 1998. 161(8): p. 3984-91.
156. Weerkamp, F., et al., Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules. Proc Natl Acad Sci U S A, 2006. 103(9): p. 3322-6.

157. Mulroy, T., Y. Xu, and J.M. Sen, beta-Catenin expression enhances generation of mature thymocytes. Int Immunol, 2003. 15(12): p. 1485-94.
158. Gounari, F., et al., Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat Immunol, 2001. 2(9): p. 863-9.
159. Goux, D., et al., Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. Blood, 2005. 106(5): p. 1726-33.
160. Xu, Y., et al., Deletion of beta-catenin impairs T cell development. Nat Immunol, 2003. 4(12): p. 1177-82.
161. Ioannidis, V., et al., The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. Nat Immunol, 2001. 2(8): p. 691-7.
162. Gounari, F., et al., Loss of adenomatous polyposis coli gene function disrupts thymic development. Nat Immunol, 2005. 6(8): p. 800-9.
163. Guo, Z., et al., Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. Blood, 2007. 109(12): p. 5463-72.
164. Dervovic, D. and J.C. Zuniga-Pflucker, Positive selection of T cells, an in vitro view. Semin Immunol, 2010. 22(5): p. 276-86.
165. Huang, Z., et al., Transcriptional regulation of CD4 gene expression by T cell factor-1/beta-catenin pathway. J Immunol, 2006. 176(8): p. 4880-7.
166. Yu, Q. and J.M. Sen, Beta-catenin regulates positive selection of thymocytes but not lineage commitment. J Immunol, 2007. 178(8): p. 5028-34.
167. Yu, Q., M. Xu, and J.M. Sen, Beta-catenin expression enhances IL-7 receptor signaling in thymocytes during positive selection. J Immunol, 2007. 179(1): p. 126-31.
168. Terra, R., et al., T-cell generation by lymph node resident progenitor cells. Blood, 2005. 106(1): p. 193-200.
169. Heinonen, K.M., et al., Wnt4 enhances murine hematopoietic progenitor cell expansion through a planar cell polarity-like pathway. PLoS One, 2011. 6(4): p. e19279.
170. Louis, I., et al., The signaling protein Wnt4 enhances thymopoiesis and expands multipotent hematopoietic progenitors through beta-catenin-independent signaling. Immunity, 2008. 29(1): p. 57-67.
171. Heinonen, K.M., et al., Wnt4 regulates thymic cellularity through the expansion of thymic epithelial cells and early thymic progenitors. Blood, 2011. 118(19): p. 5163-73.
172. Weber, B.N., et al., A critical role for TCF-1 in T-lineage specification and differentiation. Nature, 2011. 476(7358): p. 63-8.
173. Germar, K., et al., T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. Proc Natl Acad Sci U S A, 2011. 108(50): p. 20060-5.
174. Rothenberg, E.V., et al., Hematopoiesis and T-cell specification as a model developmental system. Immunol Rev, 2016. 271(1): p. 72-97.
175. Rothenberg, E.V., Transcriptional control of early T and B cell developmental choices. Annu Rev Immunol, 2014. 32: p. 283-321.
176. Anderson, M.K., et al., Definition of regulatory network elements for T cell development by perturbation analysis with PU.1 and GATA-3. Dev Biol, 2002. 246(1): p. 103-21.
177. Rothenberg, E.V., et al., Transcriptional establishment of cell-type identity: dynamics and causal mechanisms of T-cell lineage commitment. Cold Spring Harb Symp Quant Biol, 2013. 78: p. 31-41.
178. Hosoya, T., et al., GATA-3 is required for early T lineage progenitor development. J Exp Med, 2009. 206(13): p. 2987-3000.
179. Scripture-Adams, D.D., et al., GATA-3 dose-dependent checkpoints in early T cell commitment. J Immunol, 2014. 193(7): p. 3470-91.

180. Taghon, T., M.A. Yui, and E.V. Rothenberg, Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. Nat Immunol, 2007. 8(8): p. 845-55.
181. Li, L., M. Leid, and E.V. Rothenberg, An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. Science, 2010. 329(5987): p. 89-93.
182. Li, L., et al., A far downstream enhancer for murine Bcl11b controls its T-cell specific expression. Blood, 2013. 122(6): p. 902-11.
183. Yui, M.A. and E.V. Rothenberg, Developmental gene networks: a triathlon on the course to T cell identity. Nat Rev Immunol, 2014. 14(8): p. 529-45.
184. Kueh, H.Y., et al., Asynchronous combinatorial action of four regulatory factors activates Bcl11b for T cell commitment. Nat Immunol, 2016. 17(8): p. 956-65.
185. Bain, G. and C. Murre, The role of E-proteins in B- and T-lymphocyte development. Semin Immunol, 1998. 10(2): p. 143-53.
186. Bain, G., et al., Positive and negative regulation of V(D)J recombination by the E2A proteins. J Exp Med, 1999. 189(2): p. 289-300.
187. Bain, G., et al., E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. Mol Cell Biol, 1997. 17(8): p. 4782-91.
188. Engel, I., et al., Early thymocyte development is regulated by modulation of E2A protein activity. J Exp Med, 2001. 194(6): p. 733-45.
189. Tiemessen, M.M., et al., The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas. PLoS Biol, 2012. 10(11): p. e1001430.
190. Fodde, R. and T. Brabletz, Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol, 2007. 19(2): p. 150-8.
191. Muller-Tidow, C., et al., Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. Mol Cell Biol, 2004. 24(7): p. 2890-904.
192. Ysebaert, L., et al., Expression of beta-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. Leukemia, 2006. 20(7): p. 1211-6.
193. Skokowa, J., et al., LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med, 2006. 12(10): p. 1191-7.
194. Griffiths, E.A., et al., Acute myeloid leukemia is characterized by Wnt pathway inhibitor promoter hypermethylation. Leuk Lymphoma, 2010. 51(9): p. 1711-9.
195. Martin, V., et al., Methylation status of Wnt signaling pathway genes affects the clinical outcome of Philadelphia-positive acute lymphoblastic leukemia. Cancer Sci, 2008. 99(9): p. 1865-8.
196. Martin, V., et al., Epigenetic regulation of the non-canonical Wnt pathway in acute myeloid leukemia. Cancer Sci, 2010. 101(2): p. 425-32.
197. Suzuki, R., et al., Preferential hypermethylation of the Dickkopf-1 promoter in core-binding factor leukaemia. Br J Haematol, 2007. 138(5): p. 624-31.
198. Valencia, A., et al., Wnt signaling pathway is epigenetically regulated by methylation of Wnt antagonists in acute myeloid leukemia. Leukemia, 2009. 23(9): p. 1658-66.
199. Ying, J., et al., WNT5A is epigenetically silenced in hematologic malignancies and inhibits leukemia cell growth as a tumor suppressor. Blood, 2007. 110(12): p. 4130-2.
200. Minke, K.S., et al., Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. Eur J Haematol, 2009. 82(3): p. 165-75.
201. Fiskus, W., et al., Pre-clinical efficacy of combined therapy with novel beta-catenin antagonist BC2059 and histone deacetylase inhibitor against AML cells. Leukemia, 2015. 29(6): p. 1267-78.
202. Heidel, F.H., et al., Evolutionarily conserved signaling pathways: acting in the shadows of acute myelogenous leukemia's genetic diversity. Clin Cancer Res, 2015. 21(2): p. 240-8.

203. Ma, S., et al., SKLB-677, an FLT3 and Wnt/beta-catenin signaling inhibitor, displays potent activity in models of FLT3-driven AML. Sci Rep, 2015. 5: p. 15646.
204. Jamieson, C.H., et al., Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med, 2004. 351(7): p. 657-67.
205. Weerkamp, F., J.J. van Dongen, and F.J. Staal, Notch and Wnt signaling in T-lymphocyte development and acute lymphoblastic leukemia. Leukemia, 2006. 20(7): p. 1197-205.
206. Xu, M., et al., Beta-catenin expression results in p53-independent DNA damage and oncogene-induced senescence in prelymphomagenic thymocytes in vivo. Mol Cell Biol, 2008. 28(5): p. 1713-23.
207. Yu, S., et al., The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. Immunity, 2012. 37(5): p. 813-26.
208. Spaulding, C., et al., Notch1 co-opts lymphoid enhancer factor 1 for survival of murine T-cell lymphomas. Blood, 2007. 110(7): p. 2650-8.
209. Ng, O.H., et al., Deregulated WNT signaling in childhood T-cell acute lymphoblastic leukemia. Blood Cancer J, 2014. 4: p. e192.
210. Hebart, H. and H. Einsele, Clinical aspects of CMV infection after stem cell transplantation. Hum Immunol, 2004. 65(5): p. 432-6.
211. Loren, A.W., et al., Post-transplant lymphoproliferative disorder: a review. Bone Marrow Transplant, 2003. 31(3): p. 145-55.
212. Legrand, N., et al., Human thymus regeneration and T cell reconstitution. Semin Immunol, 2007. 19(5): p. 280-8.