The role of Notch and GATA3 in postnatal and adult haematopoiesis

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Abstract

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The role of Notch in cell fate determination and lineage restriction in the bone marrow (BM) is controversial in the field. Recent studies have convincingly shown that Notch is dispensable for haematopoietic stem cell (HSC) regulation in adult haematopoiesis (Maillard et al., 2008). In contrast, Notch signaling has been proposed to be of importance in the regulation of BM megakaryocyte progenitor differentiation, based on dominant negative genetic approaches, identifying a potentially distinct role for Notch in adult BM haematopoiesis (Mercher et al., 2008). Here, I found that by selectively ablating the gene coding the transcription factor recombination signal-binding protein J kappa (RBP-Jk), to which all canonical Notch signaling converges, canonical Notch signaling does not mediate HSC maintenance, neither in steady state nor in conditions of stress. Furthermore, I propose, in contrast with previous studies (Mercher et al., 2008), that canonical Notch signaling plays no role in myeloerythropoiesis cell lineage commitment in the BM. My data also show that key Notch target genes are suppressed by RBP-Jk, as

their expression is unaffected in *Notch1*-deficient BM progenitors, while target genes are upregulated in *Rbp-Jk*-deleted megakaryocyte and erythroid progenitors. This establishes for the first time in mammalian cells *in vivo,* that Notch target genes are kept in a suppressed state by RBP-Jk, potentially restricting T cell commitment to the thymus and not to the BM, at the expense of myeloerythropoiesis.

Notch signaling and GATA3 are two master regulators in T cell commitment (Han et al., 2002; Ho et al., 2009; Pui et al., 1999; Radtke et al., 1999; Zhu et al., 2004). However, although very well established as being involved in the thymic stages of T cell restriction, there is little evidence of Notch and GATA3 being involved in the migration of a thymus settling progenitor (TSP) from the BM to the thymus or in the establishment of the earliest thymic progenitor (ETP) in the thymus. From this thesis work, I conclude that Notch signaling is essential for the emergence of ETPs in the thymus in a NOTCH1-independent manner. Moreover, I demonstrate, as supported by a very recent published study (Hosoya et al., 2009), that GATA3 is important for the development of the earliest T cell progenitor.

GATA1 and GATA2 mediate haematopoietic stem cell maintenance in the BM. GATA1 is required for erythropoiesis, megakaryocytes and eosinophils while GATA2 is important for the proliferation and survival of HSCs. In contrast, a role for GATA3 in the BM has never been established. By using a *Gata3*-conditional knockout mouse model, I demonstrate that GATA3 is dispensable for HSC

maintenance in steady state and following active haematopoietic regeneration as well as for HSC self-renewal in the BM.

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List of abbreviations

µm	*micrometer*
AGM	*Aorta-gonad-mesonephros*
bHLH	*Basic helix-loop-helix*
BM	*Bone marrow*
cDNA	*Complementary DNA*
Cebp-α	*CCAAT/Enhancer-binding protein-alpha*
CFU-S	*Colony forming unit-spleen*
cGy	*CentiGray*
CLP	*Common lymphoid progenitor*
CMJ	*Cortico-medullary junction*
CMP	*Common myeloid progenitor*
CtBP	*C-terminal binding protein*
cTEC	*Cortical thymic epithelial cell*
CtIP	*CtBP interacting protein*
CTP	*Circulating T cell progenitor*
D	*Drosophila*
Dtx1	*Deltex1*
Dll	*Distal-less*
Dll1	*Delta-like 1 ligand*

Dll3	*Delta-like 3 ligand*
Dll4	*Delta-like 4 ligand*
DN	*Double negative T cell*
DNA	*Deoxyribonucleic acid*
DP	*Double positive T cell*
Dve	*Defective proventriculus*
E	*Erythrocyte*
E	*Embryonic day*
Ebf	*Early B cell factor*
EC	*Endothelial cell*
EDTA	*Ethylenediamine tetraacetic acid*
EGFP	*Enhanced green fluorescent protein*
ELP	*Early lymphoid progenitor*
Epor	*Erythropoietin receptor*
ESC	*Embryonic stem cell*
ETP	*Earliest thymic progenitor*
Etv6/Tel-1	*ETS variant gene 6*
FACS	*Fluorescence-activated cell sorting*
FL	*Flt3 ligand*
Fli1	*Friend leukaemia virus integration 1*
Flt3	*Fms-like tyrosine 3 receptor*
Fog1	*Zink finger protein multitype 1*

G	*Granulocyte*
GATA	*Gata-binding protein*
G-csfr	*Granulocyte-colony stimulating factor receptor*
Gm-csfr	*Granulocyte-monocyte-colony stimulating factor receptor*
GMP	*Granulocyte-monocyte progenitor*
Hes	*Hairy/Enhancer of split*
Hey/Hesr	*Hes-related*
Hprt	*Hypoxanthine guanine phosphoribosyl transferase 1*
HSC	*Haematopoietic stem cell*
Id2	*Inhibitor of DNA binding 2*
IFN-α	*Interferon-alpha*
Kb	*Kilo-base*
Klf1	*Kruppel-like factor 1*
Lin	*Lineage*
LMPP	*Lymphoid-primed multipotent progenitor*
Lrf	*Leukaemia/Lymphoma related factor*
LSK	*$Lin^{-}SCA\text{-}1^{+}c\text{-}KIT^{+}$*
LTC-IC	*Long-term culture-initiating cell*
LT-HSC	*Long-term haematopoietic stem cell*
M	*Macrophage*
MEP	*Megakaryocyte-erythroid progenitor*
MHC	*Major histocompatibility Complex*

Mk	*Megakaryocyte*
Mpl	*Myeloproliferative leukaemia vírus oncogene*
Mpo	*Myeloperoxidase*
MPP	*Multipotent progenitor*
mRNA	*Messenger ribonucleic acid*
mTEC	*Medullary thymic epithelial cell*
NECD	*Notch extracellular domain*
NEXT	*Notch extracellular truncated intermediate*
Nfe2	*Nuclear factor erythroid 2*
Nic	*Nicastrin*
NICD	*Notch intracellular domain*
Nk	*Natural killer*
NTMD	*Notch transmembrane domain*
PAS	*Para-aortic-splanchnopleura*
PSGL-1	*P-selectin glycoprotein ligand-1*
Psn	*Presinilin*
pTα	*Pre TCR-alpha*
PU.1	*Spleen focus forming vírus (SFFV) proviral integration oncogene*
Q-PCR	*Quantitative – polymerase chain reaction*
Rag	*Recombination-activating gene*
RAM	*RBP-Jk association module domain*

Rbp-Jk	*Recombination signal-binding protein for immunoglobulin kappa J region*
Runx	*Runt-related transcription factor*
SCF	*Stem cell factor*
Scl	*Stem cell leukaemia haematopoietic transcription factor*
SP	*Single positive T cells*
ST-HSC	*Short-term haematopoietic stem cell*
Su(H)	*Surpressor of Hairless*
TCR	*T cell receptor*
TEC	*Thymic epithelial cell*
TEPC	*Thymic epithelial progenitor cell*
Th2	*T helper 2 cell*
TNF-α	*Tumor necrosis factor – alpha*
TSP	*Thymus-settling progenitor*
Vcam-1	*Vascular cell adhesion molecule-1*
Vwf	*Von Willebrand factor*
Wg	*Wingless*
YFP	*Yellow fluorescent protein*

1

Introduction

1.1 Haematopoiesis

Haematopoiesis is the formation of mature blood cells from HSCs, that on cell division give rise to an identical daughter cell (symmetric division) or a cell with a more restricted developmental potential (asymmetric division) (Kee, 2009). Due to their fast turnover, mature blood cells need to be replenished continuously, with an estimation of one billion cells per day needed to maintain steady-state haematopoiesis in humans (Ogawa, 1993).

Mature blood cells can be divided into two distinct groups: the myeloid group and the lymphoid group. The myeloid group encompasses granulocytes (G) and macrophages (M), that are responsible for innate immunity and inflammatory responses, megakaryocytes (Mk)/platelets that promote blood clotting, and erythrocytes (E) that deliver oxygen to the tissues. The lymphoid group contains natural killer (Nk) cells, that defend the organism against tumors and virally infected cells, and T cells and B cells, involved in adaptive immunity (Kee, 2009).

1.1.1 Emergence of haematopoietic stem cells

The first blood cell emerges during embryo formation. Different classes of embryonic blood cells have been identified and their properties are specified according to the anatomical site and the time they appear during embryonic development (Dzierzak and Speck, 2008; Ottersbach et al., 2009).

In mice, haematopoiesis starts in the yolk sac at embryonic day 7.5 (e7.5) with the appearance of primitive erythrocytes and macrophage progenitors (Palis et al., 1999) (Figure 1.1). Embryonic macrophage progenitors are very proliferative

and mature very fast (Naito et al., 1989). Primitive erythrocytes express embryonic haemoglobin, crucial to the efficient oxygenation of the tissues during the fast growth of the fetus at this stage of development. These primitive haematopoietic cells are thought to derive from haemangioblasts, bipotent progenitors for the endothelial and haematopoietic cell lineages (Choi et al., 1998; Fehling et al., 2003; Huber et al., 2004; Palis et al., 1999). Around day e8-8.5, other myeloid progenitors emerge in the yolk sac, allantois and para-aortic-splanchnopleura region (PAS, to become aorta-gonad-mesonephros, AGM) (Alvarez-Silva et al., 2003; Godin et al., 1995; Palis et al., 1999). Although mature B cells are not detected in the embryo before day e17 (Delassus et al., 1998), progenitors with lymphoid and myeloid potential are found in the yolk sac and PAS at day e8.5 (Cumano et al., 1996; Godin et al., 1995).

Definitive haematopoiesis is established by the emergence of HSCs. Although their origin in the embryo is still debated in the field (Dzierzak and Speck, 2008), the first HSCs capable of repopulating all the blood lineages of adult recipients after transplantation are found in the AGM region, at day e10.5 (de Bruijn et al., 2000; Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994) (Figure 1.1). The generation of HSCs in this anatomical region has been suggested to be autonomous from the blood cells in the yolk sac, and therefore cells generated in these two different embryonic sites might have distinct progenitors (Medvinsky and Dzierzak, 1996).

There is increasing evidence for the existence of haemogenic endothelium cells, which are specialized endothelial cells capable of giving rise to haematopoietic cells (Dzierzak and Speck, 2008; Ottersbach et al., 2009).

Supporting the existence of these cells are studies showing that clusters of cells in the ventral endothelium of the dorsal aorta co-express endothelial and haematopoietic markers at the time of emergence of HSCs (Dzierzak and Speck, 2008). Therefore, the first HSCs express endothelial cell surface markers that are lost later in the foetal liver (Dzierzak and Speck, 2008). A more recent study shows that the transcription factor RUNX1 is important in the endothelial cells for the formation of HSCs from the vasculature, further supporting the existence of the haemogenic endothelium (Chen et al., 2009).

Importantly, cells capable of reconstituting neonatal mice have been described in the yolk sac and PAS around day e9 in the embryo, before HSCs are generated in the AGM region. However, since circulation in the embryo is established around day 8.25-e8.5, cells might migrate through the bloodstream from one embryonic site to another and therefore their origin remains uncertain (Figure 1.1) (Yoder et al., 1997a; Yoder et al., 1997b). Furthermore, studies using *Ncx1* mutant mice that lack heartbeat (Koushik et al., 2001) have shown evidence for the existence of cells expressing CD41 and RUNX1 in the yolk sac, two markers for definitive haematopoiesis, in the absence of circulation (Mikkola et al., 2003; Rhodes et al., 2008). But again, migration of these cells from one place to another within the embryonic vasculature cannot be excluded. In addition, no *in vivo* studies were performed to investigate whether these cells comprise the definitive HSC defining properties. Importantly, the capacity of neonatal-reconstituting cells to reconstitute adult mice is very limited if at all and therefore defining them as definitive HSCs must be taken with caution (Cumano et al., 2001; Yoder et al., 1997a; Yoder et al., 1997b).

After day e10.5, HSCs are found in the yolk sac, placenta and foetal liver. Evidence is lacking that these cells are generated *de novo* at these sites (Gekas et al., 2005; Medvinsky and Dzierzak, 1996; Muller et al., 1994; Ottersbach and Dzierzak, 2005) and HSCs are very likely to be transported from the AGM region to the sites mentioned above, through the circulation (Dzierzak and Speck, 2008).

The foetal liver becomes an active haematopoietic site from late day e9, with colony forming unit-spleen (CFU-S) activity detected at this developmental stage (Medvinsky et al., 1993). The colonization of the foetal liver by HSCs occurs at day e11 (Kumaravelu et al., 2002). However, it appears that the quantity of HSCs here is too vast to have been originated exclusively from the AGM region. Thus, it is proposed that more HSCs come from other sites that have been colonized previously, such as the yolk sac and placenta and also that HSCs must expand in the foetal liver, contributing to the increased HSC numbers in this tissue (Kumaravelu et al., 2002) (Figure 1.1).

After having colonized the foetal liver, HSCs and haematopoietic progenitors go on spreading in the embryo, reaching the thymus, spleen and finally the bone marrow (BM) around day e15, where definitive haematopoiesis occurs after birth and throughout adult life in the mouse (Dzierzak and Speck, 2008) (Figure 1.1).

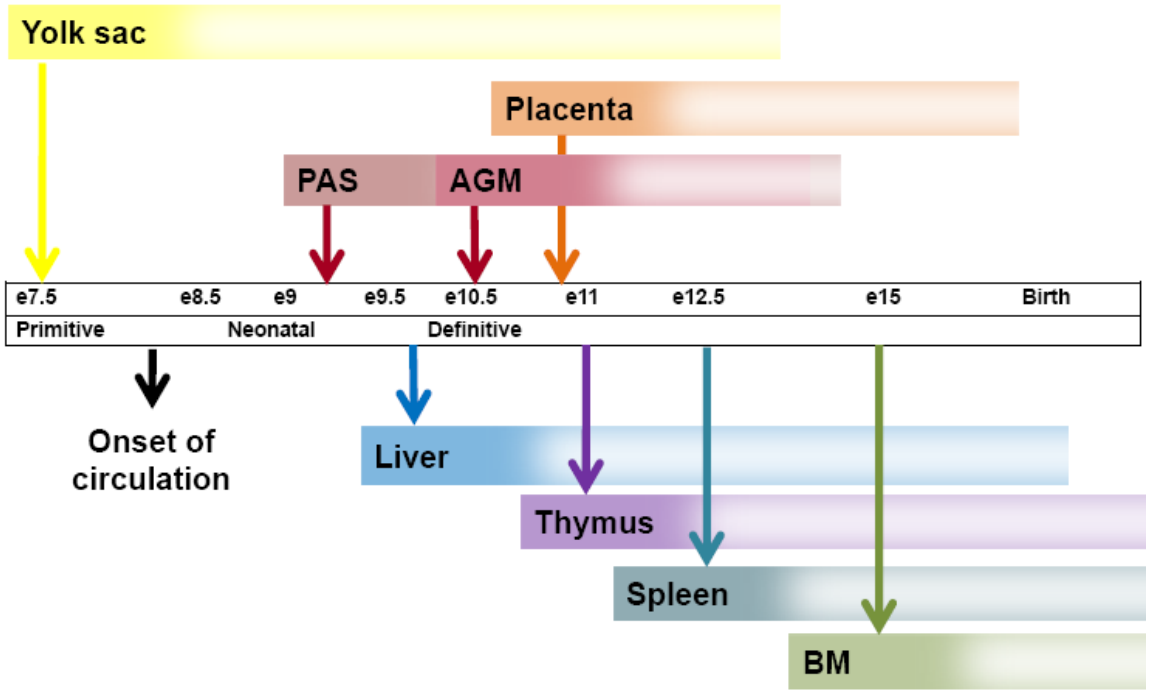


Figure 1.1. Timeline of emergence and development of haematopoiesis in the mouse embryo. Arrows above show the time and place of generation or appearance of primitive and definitive (neonatal and adult repopulating cells) haematopoietic cells. Arrows below represent where and when definitive HSCs and/or haematopoietic progenitors spread in the embryo. e: embryonic day; AGM: Aorta-gonad-mesonephros; PAS: Para-aortic-splanchnopleura, BM: bone marrow. Adapted from (Dzierzak and Speck, 2008).

1.1.2 Embryonic T cell development

The thymus is a specialized organ that exclusively supports T cell development, in both embryonic and adult life (Miller and Osoba, 1967). Although some reports have suggested a dual origin for the thymus from pharyngeal

endoderm and ectoderm (Cordier and Haumont, 1980; Cordier and Heremans, 1975; Manley and Blackburn, 2003), more recent studies (Gordon et al., 2004), established that thymus primordial arise from the third pharyngeal pouch endoderm at around day e11. Each thymus epithelial primordium is surrounded by an outer layer of neural crest cell mesenchyme that supports its growth and development (Auerbach, 1960; Jiang et al., 2000; Le Lievre and Le Douarin, 1975). At day e12.5 thymus primordial physically separate from the pharyngeal gland rudiment and migrate to the anterior part of the chest cavity (Manley and Capecchi, 1995; Ohnemus et al., 2002). Here, the thymus anlage grows and starts organizing into two main regions: the cortex, responsible for the initial differentiation of T cell progenitors, and the medulla, where final maturation steps in T cell development take place (Rodewald, 2008). A thymic epithelial progenitor cell (TEPC) has been proposed to generate cortical (cTECs) and medullary thymic epithelial cells (mTECs) (Blackburn et al., 1996; Klug et al., 1998). However, experimental tools available to purify and characterize this progenitor have been limited. Nevertheless, a $MTS20^{+}MTS24^{+}K8^{+}K5^{+}$ TEPC negative for mature cortical and medullary TEC markers and capable of generating cortical and medullary TECs has been proposed (Bennett et al., 2002; Blackburn et al., 1996; Gill et al., 2002; Klug et al., 1998; Klug et al., 2002). Subsequently, the single cell potential of TEC progenitors at the clonal level has been investigated, where single day e12.5 epithelial cells have been transplanted and gave rise to both mTECs and cTECs (Bleul et al., 2006; Rossi et al., 2006).

Loss-of-function studies and molecular studies have unveiled a novel list of genes affecting embryonic thymus development. The transcription factors

homeobox A3 (*Hoxa3)* (Manley and Capecchi, 1998)*, paired box gene 1 (Pax1) (Wallin et al., 1996), Pax3 (Conway et al., 1997; Griffith et al., 2009), Pax9 (Hetzer-Egger et al., 2002; Peters et al., 1998)*, *eyes absent 1 homologue (Eya1) (Xu et al., 2002; Zou et al., 2006), sine oculis-related homeobox 1 homologue (Six1)(Laclef et al., 2003; Zou et al., 2006)* and *T-box 1 (Tbx1)* (Jerome and Papaioannou, 2001) have been demonstrated to be involved in the initial stages of thymus organogenesis, where their absence either results in failure of initial thymus formation in the embryo, thymus aplasia, hypoplasia or impairment of thymus primordial migration to the chest cavity. Once endodermal cells have committed or differentiated into thymic epithelial cells, other critical genes become important for thymus development, where *Foxn1* plays a central role, as it is essential for thymus formation and patterning as well as TEC differentiation between day e11.5 and day e12.5. Furthermore, when *Foxn1* is absent, the colonization of the thymus primordial by lymphocyte progenitors is precluded (Blackburn et al., 1996; Gordon et al., 2001; Nehls et al., 1996; Nehls et al., 1994). In addition, *Traf6, RelB* and *p63* have also been implicated as important genes necessary for normal thymus patterning during thymus development (Akiyama et al., 2005; Burkly et al., 1995; Candi et al., 2007; Senoo et al., 2007; Weih et al., 1995; Zuklys et al., 2000).

The T-lymphoid progenitors colonize the thymus around day e10.5-11.5 in the embryo, and are first detected in the perithymic mesenchymal region at this time (Fontaine-Perus et al., 1981; Owen and Ritter, 1969). Bilateral interactions established between lymphoid progenitors and TECs are very important for the organization and maintenance of the proper cortical and medullary regions in the thymus as well as for the differentiation and maturation of T-cell progenitors

(Boehm et al., 2003; Ritter and Boyd, 1993). Importantly, recent studies demonstrated that the initial TEC differentiation events may occur in the absence of lymphoid progenitors (Klug et al., 2002). Mutant mice in which T cell development is blocked from early stages, develop normal thymus cortex and medulla compartments up to about day e15.5, from which stage TEC-thymocyte interactions are then required for thymus establishment (Klug et al., 2002). However, the mechanisms regulating the early and late stages of thymic development remain unclear.

Thymus vascularization initiates around day e12.5 (Mori et al., 2010). Pre-vascular thymus colonization is promoted by chemotactic factors secreted by the TECs that attract lymphoid progenitors from the perithymic mesenchyme into the thymus anlage (Hollander et al., 2006). In *Foxn1*-deficient mice, thymocytes accumulate in the region surrounding the thymus rudiment, establishing FOXN1 as a regulator of thymus pre-vascular colonization, promoting the accumulation of T cell progenitors within the thymus (Itoi et al., 2001; Nehls et al., 1996). Once vasculature has been established, thymocytes can enter directly into the thymus, where integrins and CD44 as well as adhesion molecules, such as the P-selectin glycoprotein ligand-1 (PSGL-1)-selectin pair, have been proposed to be important for efficient post-vascular thymus colonization (Kawakami et al., 1999; Rossi et al., 2005).

The lineage potential of the lymphoid progenitor seeding the foetal thymus has been the subject of controversy. B, T and myeloid potential found in the embryonic thymus (Hattori et al., 1996b; Peault et al., 1994) suggested that it was seeded by HSCs. However, studies failed to demonstrate HSC activity in the

thymus anlage and the presence of multipotent progenitors was not investigated (Kawamoto et al., 1998). Furthermore, in an attempt to isolate the embryonic ETP, it was shown that early thymic seeding progenitors were restricted to T and Nk cell lineages (Masuda et al., 2005) although the efficiency of the assay to support all lineages is not known. Additionally, there are studies supporting that there is T cell commitment prior to thymus colonization (Jenkinson et al., 2006; Katsura, 2002; Rodewald et al., 1994). Perithymic mesenchyme and thymic epithelium were separated prior to establishment of vascularization in the thymus to avoid contamination with blood-borne progenitors, and were separately analyzed for the presence and potential of thymic progenitors (Harman et al., 2005). Purified $CD45^+$ haematopoietic cells from both tissues generated T cells, but displayed no B cell potential (Harman et al., 2005). However, since only the CD45 surface marker was used to define haematopoietic cells, representing most likely a very heterogeneous population, B cell potential might have been missed in these studies. Notch ligands *delta-like 1* (*Dll1)* and *Dll4* were only expressed in TECs, and Notch target gene expression analysis showed that Notch signaling is not activated in mesenchymal cells (Harman et al., 2005). These data thus suggest that foetal thymic progenitors are restricted to T cell potential prior to their entry into the thymus. Moreover and in contrast with adult T cell commitment (Han et al., 2002; Radtke et al., 1999), Notch signaling seems to have no role in T cell fate choice in foetal thymopoiesis but rather supports T cell differentiation and maturation occurring after the thymus has been seeded by a T cell restricted progenitor (Harman et al., 2003; Harman et al., 2005). Importantly, other lineage potentials were not investigated in this study.

In conclusion, the identity and lineage potentials of the first progenitor seeding the embryonic thymus as well as intrathymic *versus* extrathymic Notch-mediated T cell lineage restriction in the embryo remains controversial in the field.

1.1.3 Defining adult HSCs

HSCs are mainly found in the BM of adult mammals (Kee, 2009). The definition of HSCs as it is accepted today was initially established in the early 60s by Till and McCullough, who became known as the “fathers of stem cell research” (Till and McCullough, 1961). Till and McCulloch observed that upon BM transfer into irradiated mice, blood cell colonies appeared on the spleen surface and that the number of colonies was proportional to the number of BM cells injected. Till and McCullogh then speculated that each spleen colony was derived from a single HSC (Till and McCullough, 1961). These experiments paved the way to a series of experiments that contributed to the elaboration of the two main functional criteria that a cell must meet to be identified as a stem cell. First of all, an HSC should be multipotent, thus capable of reconstituting an ablated recipient for all the blood lineages that is stable throughout the lifetime of the recipient. Secondly, an HSC should be able to self-renew, therefore capable of generating identical HSCs that maintain the stem cell pool in the BM (Kee, 2009; Till and McCullough, 1961; Weissman and Shizuru, 2008).

1.1.3.1 Prospective purification of haematopoietic stem cells

Efforts in the last two decades have successfully resulted in highly efficient prospective isolation of HSCs, mainly as a result of fluorescence-activated cell sorting (FACS) protocols (Eisenstein, 2006). In flow cytometry, fluorochrome-conjugated cells are hydro-dynamically focused in a sheath to intercept a beam of light. Upon light interception, the cells scatter the light and the fluorochromes are excited to a higher energy state, emitting a photon of light with specific spectral properties unique to different fluorochromes. Scattered and emitted light is converted to electric pulses by the optical detectors, that amplify and process these signals allowing the events (one event corresponding to one cell) to be plotted on a graphical scale. This information allows researchers to identify and characterize different subpopulations of cells based on their size and granularity (scattered light) as well as surface markers (fluorochrome emitted light). Further, cells can be physically separated based on their flow cytometric properties, a process referred to as sorting (Shapiro, 2003) (Figure 1.2).

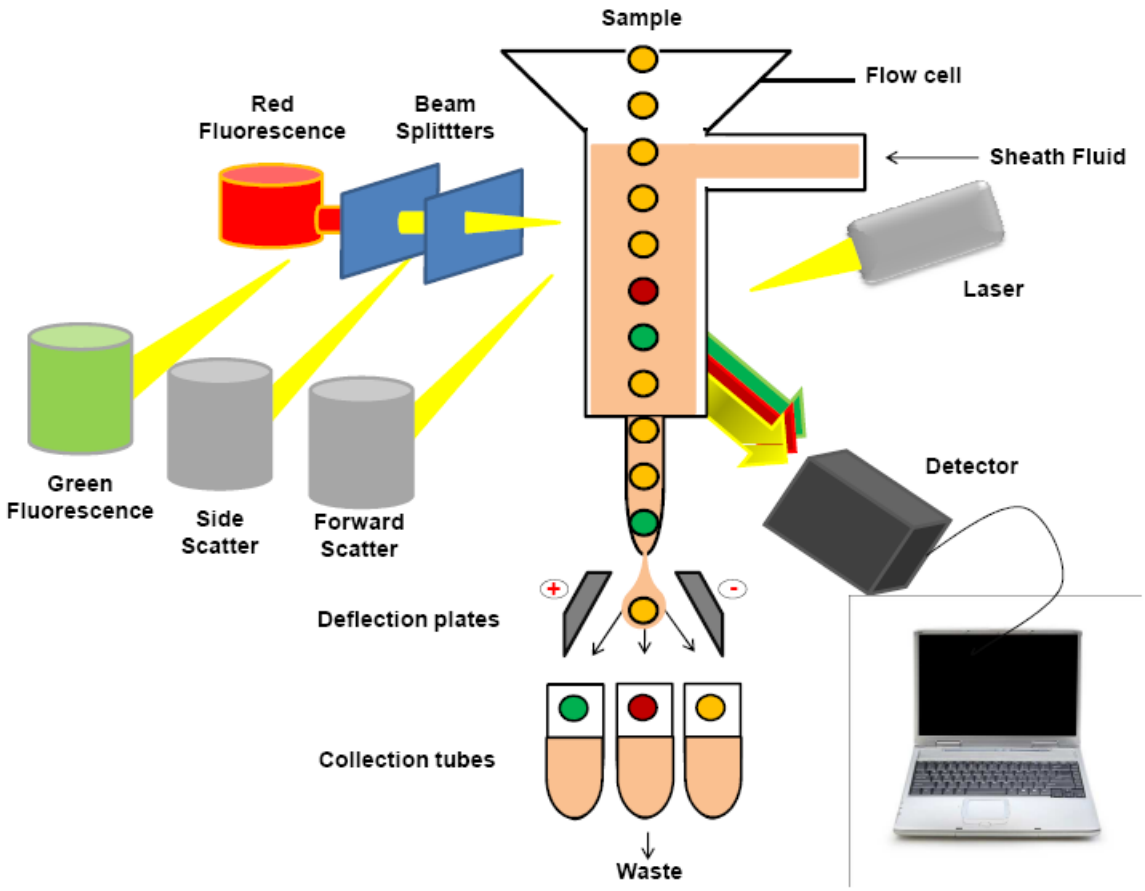


Figure 1.2. Schematic representation of the flow cytometry method (including cell sorting), outlining the major components in a flow cytometer.

HSCs are contained in the Lineage$^-$SCA-1$^+$c-KIT$^+$ (LSK) heterogeneous compartment, where mature blood cell markers are not expressed (Lineage negative; Lin$^-$) and high levels of SCA-1 and c-KIT receptor are detected on the cell surface (Ikuta and Weissman, 1992; Li and Johnson, 1995; Spangrude et al., 1988).

HSCs are very rare in the haematopoietic system, representing only 0.05% to 0.1% of total murine BM cells (Szilvassy et al., 1990), hence their isolation and purification is required to allow them to be directly studied and characterized. Given the heterogeneity of the LSK stem cell compartment, identification of further surface markers beyond SCA-1 and c-KIT became important to purify HSCs from progenitors.

HSCs can be subdivided into two functionally distinct subpopulations. The long-term HSCs (LT-HSCs) are characterized by their high capacity to self-renew and, in the adult mouse have been shown to express, in addition to SCA-1 and c-KIT (Li and Johnson, 1995; Spangrude et al., 1988; Weissman et al., 2001), the slam protein CD150 (SLAMF1) (Kiel et al., 2005), Endoglin (CD105) (Chen et al., 2002) and Thy1.1 in some mouse strains (Spangrude et al., 1988), and are negative for CD34 (Osawa et al., 1996), fms-like tyrosine 3 receptor (FLT3) (Adolfsson et al., 2001; Christensen and Weissman, 2001) and the SLAM marker CD48 (Kiel et al., 2005). The achievement of this phenotypic signature of LT-HSCs allowed purifying them to a degree that at least one in five cells resulted in long term reconstitution of recipient mice (Kiel et al., 2005; Osawa et al., 1996).

LT-HSCs differentiate into short-term HSCs (ST-HSCs), characterized by the upregulation of CD34 (Yang et al., 2005). Functionally, ST-HSCs show a more limited self-renewing capability than LT-HSCs (Reya et al., 2001; Weissman et al., 2001) and can rescue myeloablated recipients through a rapid but transient myeloid and lymphoid reconstitution (Yang et al., 2005). Upregulation of FLT3 in ST-HSCs (towards LSKCD34^{+}Flt3^{+}, known as multipotent progenitors (MPPs)) is associated with upregulation of other genetic factors that compose what is referred

to as the lymphoid gene program (Adolfsson et al., 2005; Mansson et al., 2007). Given the limitation of testing and to functionally distinguish ST-HSCs from LT-HSCs, it is important to note that the former have not to date been prospectively purified. Thus, ST-HSCs can be divided into two phenotypically and functionally distinct subpopulations based on the expression of FLT3 receptor and represent an intermediate stage in the commitment of an HSC into myeloid *versus* lymphoid progenitors (Yang et al., 2005).

1.2 Haematopoietic lineage commitment – Emerging roadmaps

A series of lineage commitment events occurs during haematopoietic differentiation (Kawamoto and Katsura, 2009; Luc et al., 2008b). HSC-derived MPPs lose multipotency and move towards a single lineage, ultimately resulting in a mature blood cell. Lineage commitment is therefore a stepwise process, thought to be regulated by intrinsic and extrinsic regulators, such as cytokines and transcription factors (Ceredig et al., 2009; Kee, 2009). It culminates in a progenitor restricted to a unique lineage upon loss of alternative fate options. Nevertheless, cellular pathways and restriction sites governing the generation of unipotent progenitors from HSCs are not consensual in the field.

1.2.1 The classical model

The first model proposed for haematopoietic lineage commitment – the classical model - implied that the first cell fate decision strictly separates lymphoid

and myeloid lineages (Figure 1.3). In this model an HSC differentiates and branches apart into the so-called common lymphoid progenitor (CLP), a progenitor phenotypically defined as $Lin^{-}IL\text{-}7R^{+}THY\text{-}1^{-}SCA\text{-}1^{lo}c\text{-}KIT^{lo}$ that lacks myeloid potential and can differentiate into Nk, T and B cells (Kondo et al., 1997), and the common myeloid progenitor (CMP; $Lin^{-}IL\text{-}7R^{-}SCA\text{-}1^{-}c\text{-}KIT^{+}$ $CD34^{+}$ $Fc\gamma R^{lo}$), capable of further committing into MkE progenitor (MEP) or GM progenitors (GMP), that, in contrast to MEP, expresses CD34 and high levels of Fcγ receptor on the cell surface. It is of note that it has recently been questioned whether $Lin^{-}IL\text{-}7R^{-}SCA\text{-}1^{-}$ $c\text{-}KIT^{+}$ $CD34^{+}$ $Fc\gamma R^{lo}$ CMPs, with mixed Mk/E/GM potential really exist and functionally distinct GM and MkE progenitors deriving from CMP have been described (Pronk et al., 2007). Furthermore, in contrast with CMPs being downstream to the LSK compartment, transcriptional priming pattern studies observed that $LSKFLT3^{-}$ HSCs express mostly GM and MkE family genes while little or no lymphoid transcriptional programs are expressed, suggesting that myeloid progenitors may emerge earlier in the hierarchy (Luc et al., 2008a; Mansson et al., 2007). Accordingly, studies in *GATA1-GFP* reporter mice established that primitive $LSKFLT3^{-}GATA\text{-}1^{+}$ cells reconstitute MkE and GM lineages very efficiently (Arinobu et al., 2007) and lack any lymphoid potential, thus representing a more primitive CMP in the stem cell compartment. In addition, myeloid potential has recently been demonstrated in CLPs (Ehrlich et al., 2010; Inlay et al., 2009).

There have been several rigorous studies demonstrating the existence of lymphoid progenitors with remaining myeloid potential thus contradicting a strict separation between lymphoid and myeloid lineages from HSCs (Adolfsson et al.,

2005; Bell and Bhandoola, 2008; Wada et al., 2008). Furthermore, the most common bi-phenotypic leukemias diagnosed, are either T-myeloid or B-myeloid, while T-B leukemias appear to be very rare (Matutes et al., 1997). Thus, the classical model needs revision.

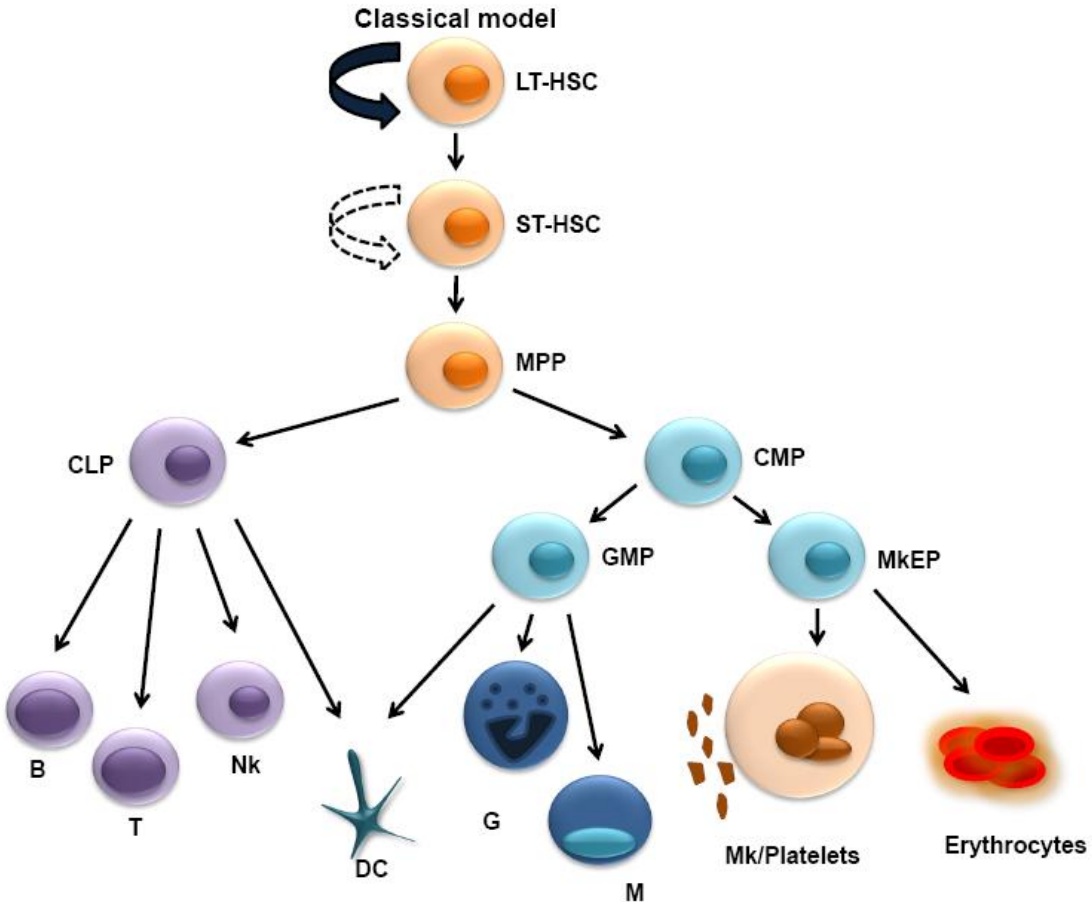


Figure 1.3. The CMP-CLP model. The classical model above infers a strict separation between myeloid and lymphoid lineages. Curved filled arrow refers to self-renewal potential of LT-HSCs and curved dashed arrow indicates the reduced self-renewal potential of ST-HSCs. LT-HSC: long-term haematopoietic stem cell; ST-HSC: short-term HSC; MPP: multipotent progenitor; CLP: common lymphoid progenitor; CMP: common

myeloid progenitor; GMP: granulocyte-monocyte progenitor; MkEP: megakaryocyte-erythrocyte progenitor; Nk: natural killer cell; DC: dendritic cell.

1.2.2 Alternative models for blood lineage commitment

The classical model implies that at any stage of haematopoietic development, a cell with concomitant lymphoid and myeloid potential should not exist. However increasing evidence argues against this strict lineage separation.

Accordingly, the CLP-CMP model was for the first time challenged by Katsura and colleagues who suggested, in 2001, what they called the myeloid-based model, for the lineage commitment in definitive foetal haematopoiesis (Katsura and Kawamoto, 2001). This model was based on studies previously published by the same group in which they developed an *in vitro* assay which allowed concomitant differentiation of B, T and myeloid cells and used it to propose the existence of T/M and B/M progenitors deriving from a M/B/T MPP that lacks E potential (Kawamoto and Katsura, 2009; Kawamoto et al., 1999; Kawamoto et al., 1997; Lu et al., 2002). However, these interpretations should be taken carefully, and interpreted as an indication rather than evidence of possible progenitor types, as a mixture of multipotent and restricted progenitor cells were studied in the same cultures and differential lineage potential readouts were interpreted as reflecting the existence of distinct progenitors with different lineage potentials (Katsura, 2002), whereas the same outcomes could have been seen simply if the assays were not 100% efficient at reading out all lineage potentials for every progenitor

investigated. Thus, a prospective isolation, purification and characterization of the different progenitors, similar to what has been done for the identification of different stages of lineage commitment in adults (Reya et al., 2001), is required to resolve the existence of these myeloid-lymphoid progenitors in the embryo. In adults, Montecino-Rodriguez *et al* some time ago suggested the existence of a $B220^{-}$ $CD19^{+}$ progenitor in the BM with B cell and macrophage potentials (Montecino-Rodriguez et al., 2001), further contesting the CLP-CMP model, also in adults.

More recently, more convincing evidence has been provided for the existence of a lymphoid-primed multipotent progenitor (LMPP) in the LSK early compartment, in both adult bone marrow (Adolfsson et al., 2005) and foetal liver (Mansson et al., 2007), that expresses high levels of FLT3 (25% FLT3 highest expressing fraction in the LSK compartment) and that, in addition to B and T cell potential, has a prominent GM potential but retains very little or no MkE potential. In fact, MkE potential is completely absent in further purified LMPPs expressing the Spleen focus forming vírus (SFFV) proviral integration oncogene product PU.1 (Arinobu et al., 2007) or lacking the Myeloproliferative leukaemia vírus oncogene (MPL) receptor (Luc et al., 2008a). At the molecular level, LMPPs sustain extensive granulocyte-monocyte gene transcriptional priming and unlike HSCs upregulate lymphoid-related genes while the Mk/E gene signature is downregulated (Adolfsson et al., 2005; Mansson et al., 2007; Yang et al., 2005). Importantly, through the usage of other surface markers, B/T/GM tripotent MPP progenitors overlapping with LMPPs were subsequently identified through alternative approaches (Lai and Kondo, 2006; Yoshida et al., 2006). Based on the existence of this B/T/GM progenitor a revised model was suggested where LMPP

sits upstream to CLP and implicating a distinct MEP or CMP progenitor branching off at a restriction site before LMPPs and just downstream to HSCs (Adolfsson et al., 2005; Luc et al., 2008b) (Figure 1.4). The existence of such a primitive CMP was subsequently identified through the use of a GATA-1 reporter (Arinobu et al., 2007). Initially, the LMPP model was questioned due to the fact that myeloablated recipients reconstituted with some erythrocytes and platelets upon transplantation of high numbers of LMPPs (Forsberg et al., 2006). However, the same study reported that only a very small number of LMPPs could form spleen colony-forming units as an indicative of E potential *in vivo*. Thus, this study too was compatible with most LSKFlt3^{hi} cells having little or no MkE potential (Forsberg et al., 2006) further corroborating the previous finding that these cells have at most 2-3% MkE potential (Adolfsson et al., 2005) (Figure 1.4).

1.2.3 A T/GM progenitor in the thymus

Recently, a progenitor with combined T and myeloid (GM) potential has been identified in the thymus (Bell and Bhandoola, 2008; Wada et al., 2008). Through *in vitro* clonal assays as well as *in vivo* settings it was demonstrated that the adult ETPs, defined as Lin$^-$CD4$^-$CD8$^-$c-KIT^{hi}CD25$^-$, are capable of generating both T and myeloid cells when cultured at the single cell level, and have already lost the B cell potential which the cell seems to have in the BM (Bell and Bhandoola, 2008; Wada et al., 2008). Importantly, one should be mindful that the assays used here might have been ineffective in evaluating a possible concomitant B cell potential of these progenitors to T/GM potentials. Nevertheless, neither of

these reports found ETPs to have B cell potential, even at low frequency, suggesting that ETPs might be T/GM restricted in their lineage potential. Importantly, a restricted T/GM progenitor has never been described in the BM, thus introducing several scenarios that could reconcile the existence of multipotent thymus seeding progenitors in the BM with thymic ETPs with T/GM potential. One possibility is that a T/GM progenitor exists in the BM but remains to be identified. Alternatively, a T/GM progenitor would be generated outside the BM while migrating to the thymus or a T/B/GM progenitor yet to be found does exist in the thymus within or outside the ETP population. However, further investigation is required to definitely support any of these hypotheses.

Overall, the latest studies estabished the existence of lymphoid progenitors that preserve myeloid potential (Figure 1.4).

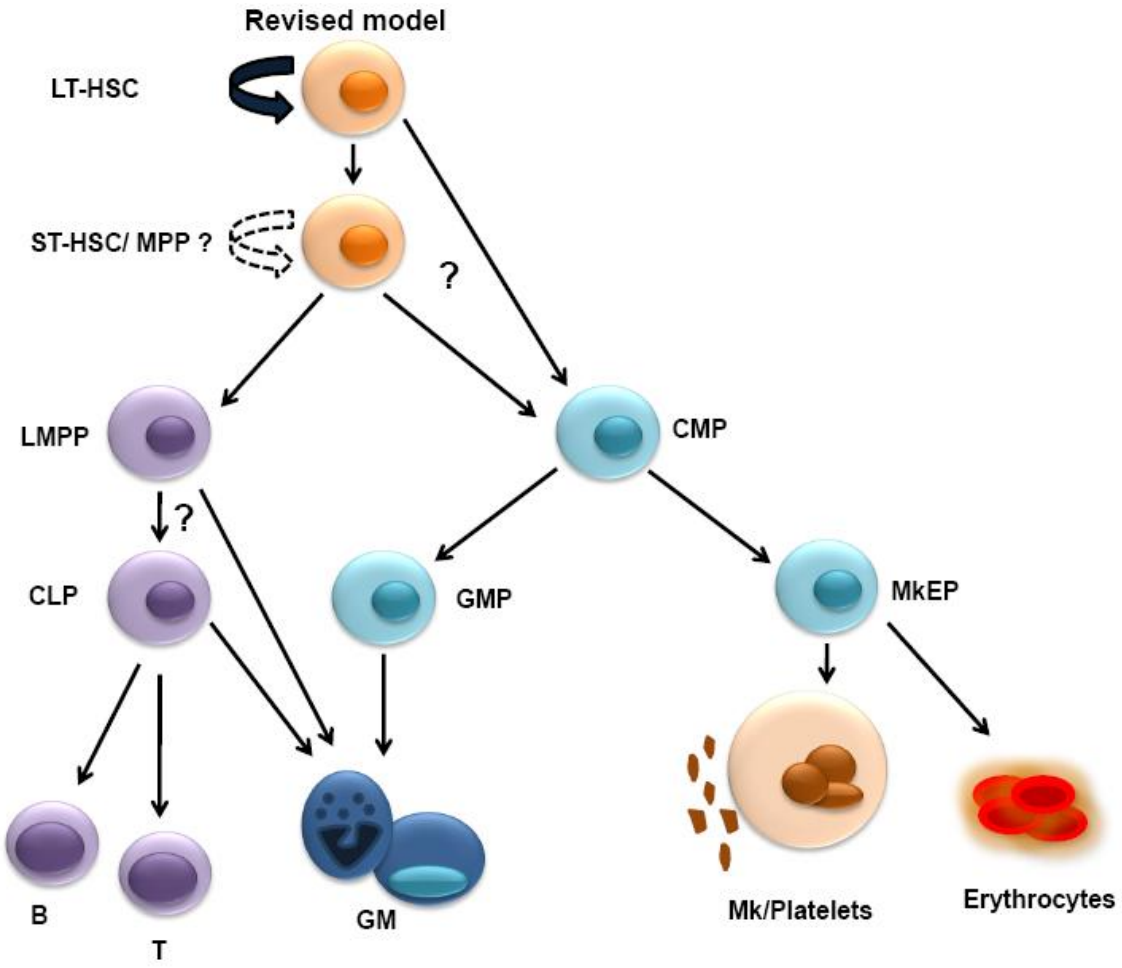


Figure 1.4. Revised model for adult haematopoiesis. In this revised model based on knowledge as of today, a maintenance of myeloid potential in lymphoid progenitors in conjectured. Question marks indicate intermediate commitment steps and/or cellular intermediates not yet fully understood or identified. Curved filled arrow refers to self-renewal potential of LT-HSCs and curved dashed arrow indicates the reduced self-renewal potential of ST-HSCs. LT-HSC: long-term haematopoietic stem cell; ST-HSC: short-term HSC; MPP: multipotent progenitor; LMPP: lymphoid-primed multipotent progenitor; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; GMP: granulocyte-monocyte progenitor; MkEP: megakaryocyte-erythrocyte progenitor.

1.2.4 Transcriptional regulation of lineage commitment in the haematopoietic hierarchy

A complex network of transcription factors regulates cell fate in haematopoiesis (Ceredig et al., 2009). The initial concept that specific transcription factors almost exclusively correspond to a given cell lineage was challenged by the observation that HSCs express genes of the different lineages at the single cell level (Hu et al., 1997; Miyamoto et al., 2002; Orkin, 2003). Furthermore, this phenomenon, termed multilineage transcriptional priming, establishes that an HSC or a multipotent progenitor retains the potential to differentiate towards any lineage, their fate being determined by repression of alternative lineage options. In addition, the co-expression of transcription factors representative of different cell lineages allows modulation of the different fate potentials within the cell at the molecular level. A classical example is the complex relationship between GATA1 and PU.1 transcription factors. High levels of *Gata1* specify MkE development from HSCs while *PU.1* represses erythroid fate favouring myelo-lymphoid lineages (Arinobu et al., 2007). Therefore, their simultaneous modulation within the cell will determine MkE *versus* myelo-lymphoid cell commitment (Arinobu et al., 2007; Monteiro et al., 2011).

Importantly, albeit multilineage primed, HSCs and primitive LSKFLT3^{-}GATA-1^{+} CMPs are primarily MkE primed, expressing concomitantly myeloid gene programs, while lymphoid priming is hardly observed (Arinobu et al., 2007; Mansson et al., 2007), with the exception of a recent study suggesting an *Ikaros*-

dependent lymphoid priming in HSCs (Ng et al., 2009). A marked priming for all lineages is observed immediately downstream, at the MPP stage (Akashi et al., 2003). LMPPs are the first early MPPs where lymphoid priming is robustly initiated with concomitant down-regulation of the MkE transcriptional program (Mansson et al., 2007). Interestingly, myeloid genes, such as *Myeloperoxidase (Mpo)* and *Granulocyte-colony stimulating factor receptor (G-csfr),* are activated in both HSC and LMPP compartments (Mansson et al., 2007). Thus, transcriptional regulation studies further support a separation between GM/MkE and GM/lymphoid lineages early in the hierarchy, where myeloid fate potential is conserved also in lymphoid progenitors, in agreement with the existence of GM-lymphoid LMPPs and GM-MkE primitive CMPs (Arinobu et al., 2007). Downstream to MPPs, lineage specific transcriptions factors start to be upregulated while other transcription factors representing alternative lineages are silenced or repressed, resulting in a progressive loss of alternative lineage potentials finally culminating in a unipotent lineage restricted progenitor (Ceredig et al., 2009).

1.2.5 Concluding remarks

In conclusion, the classical CLP-CMP model although conceptually largely correct and very important for our current understanding of haematopoiesis, does not take into account a number of recent findings incompatible with this model including evidence for a gradual and stepwise restriction of a multipotent progenitor towards a single lineage, such as the LMPP. Nevertheless, exactly how lineage commitment happens remains an open question and further investigation is

required to fully understand the cellular and molecular mechanisms governing lineage commitment in the haematopoietic system, and also to what degree these pathways are conserved between foetal and adult haematopoiesis. However, and importantly, recent findings do suggest that most of the main lineage restriction events and the myeloid-based commitment is conserved in embryonic and adult mouse (Luc et al., 2008b) as well as human haematopoiesis (Doulatov et al., 2010).

1.3 Notch signaling

Notch signaling is a highly conserved pathway between species, from worms to humans, and is involved in processes such as cell fate determination, tissue homeostasis and cell differentiation in many different organisms (Radtke et al., 2010).

1.3.1 Notch receptors and ligands in mammals

Different organisms have different numbers of Notch receptors, however all of them are composed of similar and conserved domains. In mammals, four different Notch receptors (Notch1-4) (Bray, 2006), which can bind to three distinct Delta-like ligands (Dll1, Dll3 and Dll4) and two Serrate-like ligands (JAGGED1 and JAGGED2) have been described (Kopan and Ilagan, 2009).

Notch receptors are type I transmembrane proteins and consist by an extracellular domain (NECD), with EGF-like repeats (36 in NOTCH1 and NOTCH2,

34 in NOTCH3 and 29 in NOTCH4) that mediate receptor-ligand interactions, and three cysteine-rich Notch/LIN12 repeats (LIN) that prevent ligand-independent signaling, a transmembrane domain (NTMD) followed by an intracellular domain (NICD). The NICD contains a recombination signal-binding protein for immunoglobulin Kappa J region (RBP-Jk) association module (RAM) domain, that associates with the RBP-Jk deoxyribonucleic acid (DNA)-binding protein in the nucleus, a seven ankyrin repeats (ANK) domain, nuclear localization sequences (NLS), a transactivation domain (TAD), which is absent in NOTCH3 and NOTCH4 receptors, and conserved proline/glutamic acid/serine/threonine-rich motifs (PEST), that are essential for the fast turnover of NICD in the nucleus (Bray, 2006; Kopan and Ilagan, 2009).

Notch ligands are also type I transmembrane proteins composed of an N-terminal Delta/Serrate/LAG-2 (DSL) motif followed by EGF-like repeats. JAGGED1 and JAGGED2 ligands present a cystein rich domain downstream to the EGF-like repeats. (Kopan and Ilagan, 2009).

1.3.2 The first sparkle in the Notch signaling ignition

Translated Notch proteins undergo O-fucosylation and O-glycosylation in the endoplasmatic reticulum. This process was initially thought to be essential to form a functional Notch receptor, however later studies demonstrated that this post-translational process rather causes an upregulation of chaperone activity in the cell, promoting the folding and transport of the Notch protein to the cell surface membrane (Kopan and Ilagan, 2009). The Notch polypeptide then enters the Golgi

apparatus, where the carbohydrate chains can be extended by glycosyltransferase Fringe enzymes (Lunatic, Maniac and Radical Fringe in mammals). This path has been suggested to modulate the affinity of the receptor for a given ligand to the detriment of another one (Bray, 2006; Kopan and Ilagan, 2009) and references therein).

In the secretory pathway, occurs the first proteolytic cleavage of the Notch protein at site 1 (S1) by furin-like proteases occurs. A Notch heterodimer, the Notch extracellular domain-Notch transmembrane and intracellular domain (NECD-NTMIC) results from this cleavage event and can travel to the cell surface membrane (Kopan and Ilagan, 2009) and references therein). Once on the surface, Notch receptors are exposed to neighboring cells that express and present the Notch ligands (Kopan and Ilagan, 2009).

Upon binding of the Notch ligand to the receptor, a second cleavage takes place at the NECD of the receptor (site 2 – S2). Proteolysis is mediated by two different metalloproteases - ADAM10 and tumor-necrosis factor-α (TNF- α)-converting enzyme (TALE, also known as ADAM17). A Notch extracellular truncated intermediate (NEXT) is generated becoming a substrate for the protease enzyme γ-secretase, which cleaves NEXT within the NTMD – S3, S4 cleavages. NICD is now free in the cytoplasm to translocate to the nucleus and trigger transcriptional gene activation (Bray, 2006; Kopan and Ilagan, 2009) and references therein) (Figure 1.5).

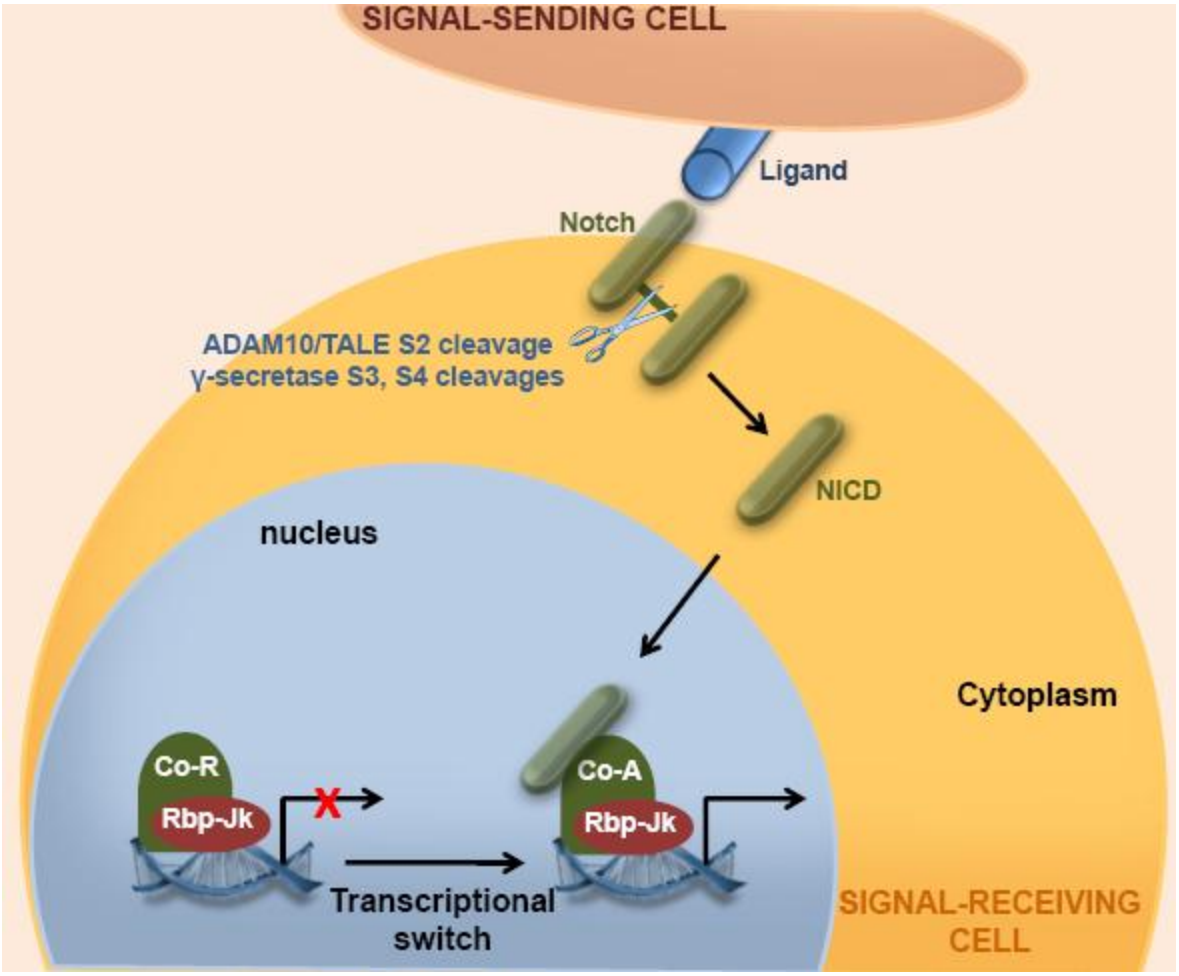


Figure 1.5. Schematic representation of the Notch signaling pathway. NICD: Notch intracellular domain, Co-R: co-repressor complex, Co-A: co-activator complex, Rbp-Jk: Recombination signal-binding protein for immunoglobulin kappa J region.

1.3.3 "Let's start the action!"

Once in the nucleus, NICD is incapable of binding to DNA. Transcriptional activation of the Notch target genes is therefore mediated by the DNA-binding transcription factor CSL (named after CBF-1 in mammals, Surpressor of Hairless in

Drosophila melanogaster and LAG-1 in *Caenorhabditis elegans*), commonly known as RBP-Jk or CBF-1 in mammals. RBP-Jk is a highly conserved protein that plays a central role in the Notch signaling pathway, where Notch signals from all four Notch receptors converge, converting the protein signaling into transcriptional regulation of gene expression. RBP-Jk is therefore activated by binding of NICD through its RAM domain (Bray, 2006; Kopan and Ilagan, 2009) (Figure 1.5).

1.3.3.1 The transcriptional repression- activation "switch"

In the absence of NICD, RBP-Jk recruits the co-repressors SHARP/SPEN/MINT or silencing mediator for retinoic acid and thyroid hormone receptor (SMRT; also known as NcoR) (Kao et al., 1998; Oswald et al., 2005), which in turn recruit the C-terminal binding protein (CtBP) and the CtBP interacting protein (CtIP) to form a repressor complex that sits on the Notch target gene promoters, keeping their activation down (Oswald et al., 2005). Two other RBP-Jk-interacting proteins, SKIP and CBF1-interacting co-repressor (CIR), have also been identified as part of the repression complex (Hsieh et al., 1999; Zhou et al., 2000) (Figure 1.5).

Upon binding of NICD to RBP-Jk, the transcriptional co-repressors are displaced and co-activators are recruited. Accordingly, ANK repeats in the NICD protein bind weakly to RBP-Jk, however the interface Rbp-Jk/ANK is very important to attract the N-terminal α-helix of the Mastermind-like family of transcriptional activators (MAML1-3/LAG-3) that stabilize the NICD/RBP-Jk heterodimer bound to DNA during the transcriptional activation of Notch target

genes. The NICD/RBP-Jk/MAML ternary complex recruits additional co-activators such as the acetyltransferase p300, chromatin remodelling factors and the Mediator complex, assembling an active transcription complex on the target promoters, initiating the expression of Notch target genes, such as the *Hairy/Enhancer of split (Hes)* and the *Hes*-related (*Hesr/Hey*) family of basic helix-loop-helix (bHLH) transcription factors (Bray, 2006; Kopan and Ilagan, 2009) (Figure 1.5).

1.3.3.2 RBP-Jk: transcriptional activator or repressor?

Since the RBP-Jk protein is a central transcriptional effector of the Notch signaling pathway, it would be reasonable to expect that loss of Notch signaling would result in downregulation of Notch target gene expression, determining RBP-Jk as an activator of Notch signaling. However, accumulating evidence has suggested that RBP-Jk functions simultaneously as a transcriptional repressor and activator, which is anyway a commonality in many highly effective signaling pathways (Bailey and Posakony, 1995; Barolo and Posakony, 2002; Dou et al., 1994; Lecourtois and Schweisguth, 1995).

1.3.3.2.1 CSL is an active repressor of Notch target genes in Drosophila

A study in *D. Melanogaster* by Bailey and Posakony reported that the transcriptional activation of Notch target genes in the E(spl) complex increases in

the absence of *Surpressor of Hairless* (Su(H)), the homologue of *Rbp-Jk* in flies (Bailey and Posakony, 1995). Furthermore, the weak or complete absence of a phenotype in *Su(H)* mutant *D. Melanogaster* flies during development of the wing and formation of the dorsoventral compartment boundary strikingly contrasts with the phenotype in *Presinilin (Psn)* and *Nicastrin (Nic) null* flies, both genes coding for proteins involved in the receptor proteolysis, where mutants down-regulate the expression of the *defective proventriculus (dve)* and *Distal-less (Dll)* Notch target genes. This discrepancy has been suggested to be due to the full loss of the Su(H) repressive function, where Notch target activation is observed in *Su(H)* null mutants, although to a weaker extent than when Notch signaling is activated, contrary to *Psn* and *Nic* mutants, where no activation of the same targets is detected (Koelzer and Klein, 2006). Additionally, this loss of Su(H) repressive function has been proved to be Notch independent, since Su(H);Psn double mutants expressed *Dll, dve* and *wingless (wg)* Notch target genes, similarly to what was observed in the *Su(H)* single mutant (Koelzer and Klein, 2006). Collectively, these results show that loss of function of Su(H) is independent of Notch signaling and the observed derepression of target genes conceals the involvement of Su(H) in developmental processes in flies (Bailey and Posakony, 1995).

Additionally, a more recent study in mouse fibroblasts has demonstrated that the Notch target gene, *Hey1,* is derepressed in the absence of CtBP, a corepressor recruited by the RBP-Jk/SHARP complex to silence Notch target genes (Oswald et al., 2005), suggesting that RBP-Jk may function as a transcriptional repressor rather than an activator of Notch target genes also in mammals. Although the *D. Melanogaster* studies support that derepression of

Notch target genes is due to a Notch-independent loss of repressive function of Su(H), further studies are required to verify that this is also the case in mammals *in vivo*. Nevertheless, these studies strongly suggest that CSL, at least in some tissues in some organisms, actively keeps Notch target genes in a suppressive state that hypothetically may influence events such as cell fate determination and commitment. However, whether and in which cell types derepression may occur in mammals remains to be shown as well as whether this repressive function of RBP-Jk is or is not a Notch-independent event.

1.3.4 Non-canonical Notch signaling

The CSL-dependent Notch signaling is very well established as a master signaling pathway in many cellular processes, such as cell fate determination, development or homeostasis in many cellular contexts and is widely supported by numerous studies in various tissues and cell types of different organisms (Lai, 2004). Nevertheless, some evidence has emerged for the existence of non-canonical Notch signaling.

Studies in *Drosophila* embryos demonstrated that the phenotype of the only *Notch receptor and Su(H)* mutants were not identical, but rather stronger in the *Notch* mutants (Rusconi and Corbin, 1998, 1999; Zecchini et al., 1999). In vertebrates, evidence supporting the existence of non-canonical Notch signaling is less clear. In 1996, Shawber and colleagues overexpressed *Notch1* in muscle cells and observed that cell differentiation was inhibited without interaction of NOTCH1 with CSL or upregulation of the Notch target gene, *Hes1* (Shawber et al.,

1996). Further studies have since then been reported suggesting the occurrence of CSL-independent Notch signaling in cellular processes such as development of the peripheral nervous system and neural crest, or axon growth and guidance (Talora et al., 2008).

Initial studies reported the existence of RBP-Jk-independent Notch signaling and Notch target gene activation, where NICD interacts with components of other signaling pathways to activate Notch target genes (Sanalkumar et al., 2010a). Experimentally, these studies were based on approaches where Notch receptors lacked the RBP-Jk binding domain, RAM, or *Rbp-Jk null* cell lines and mouse models were used (Sanalkumar et al., 2010a). In these studies, RBP-Jk independent Notch targets activation was either mediated upon Notch ligand-receptor interaction that triggered NICD cleavage or via Notch receptor cleavage-independent target genes activation. Regardless of whether dependent or independent of NICD to trigger gene activation, RBP-Jk-independent non-canonical Notch signaling networks works together with signaling pathways such as Hedgehog, Jak/signal transducer and activator (STAT), receptor tyrosine kinase (RTK), transforming growth factor (TGF)-β and wingless-type MMTV integration site (Wnt) to impact on different cell functions such as cell fate determination, proliferation, stem cell maintenance and oncogenesis (Barolo and Posakony, 2002; Cummings, 2006; Pires-daSilva and Sommer, 2003). There has also been reports of Notch-independent but RBP-Jk-dependent non-canonical Notch signaling, in which the target genes, *Hes1* and *Hes5,* can be regulated in cancer and retinal cells respectively in a cellular context devoid of NICD cleavage but in an RBP-Jk-dependent manner (Hori et al., 2008; Stockhausen et al., 2005).

In addition, some Notch target genes can be regulated independently of Notch/CSL interaction, such as *Hes1* in neural progenitors (Sanalkumar et al., 2010b), retinal progenitors (Wall et al., 2009) and haematopoietic progenitors (Ikawa et al., 2006; Maillard et al., 2008), among other cell types.

It should not be ruled out that canonical and non-canonical Notch signaling can happen simultaneously or alternately, with distinct functions in cell development and maintenance. Moreover, the mechanism through which the signals propagate might differ from canonical to non-canonical pathways, such as the events of cleavage of the Notch receptor or translocation of NICD to the nucleus.

Nevertheless, it should be noted that biochemical mechanisms for an RBP-Jk independent Notch signaling pathway in mammals remain to be fully understood.

1.4 Notch signaling in bone marrow haematopoiesis

1.4.1 Role of Notch in HSC self-renewal and maintenance

Notch signaling is very important in cell fate determination in various organisms (Lai, 2004). In the BM, Notch receptors and ligands are all detectable although at much lower levels than in the thymus (Felli et al., 1999; Kojika and Griffin, 2001). Moreover, NOTCH1 has been shown to be essential for the generation of HSCs, most likely from haemogenic endothelium in the embryo (Kumano et al., 2003). These observations marked out Notch signaling as a

candidate regulator of adult HSC generation and maintenance, leading to several gain- and loss-of-function studies trying to establish Notch as functionally important in adult HSCs.

1.4.1.1 Gain-of-function studies support Notch as an important regulator of HSC self-renewal

Initial studies showed that haematopoietic cell lines overexpressing NICD could no longer differentiate in response to cytokines (Bigas et al., 1998; Milner et al., 1996). These studies were further supported by evidence of constitutive Notch1 signaling in BM progenitors resulting in the generation of a cytokine-dependent cell line capable of differentiating into all the different blood lineages (Varnum-Finney et al., 2000) and reconstituting transplanted mice, although an extremely high number of donor cells were required to reconstitute the mice at very low levels (Stier et al., 2002). Additionally, HSC differentiation was arrested upon Notch signaling activation in BM progenitors when exposed to immobilized DELTA1 ligand *in vitro* (Varnum-Finney et al., 2003). Furthermore, overexpression of the Notch target gene, *Hes1,* in a purified LSKCD34$^-$ HSC-comprising population results in an increased long-term repopulating capacity in *in vitro* long-term culture-initiating cell (LTC-IC) cultures as well as increased ratios of HSCs derived from *Hes1*-transduced LSKs when compared to control recipients in a competitive transplantation assay (Kunisato et al., 2003). Further corroborating an effect of Notch signaling in inhibiting HSCs differentiation and keeping their self-renewal capacity is a study from Calvi and colleagues in which mice induced to express

high levels of *Jagged1* have increased numbers of HSCs resulting from activation of NOTCH1 *in vivo* (Calvi et al., 2003). Although these studies support that Notch has an effect on HSC self-renewal and maintenance in an undifferentiated state, the physiological role of Notch in regulation of HSCs has not been investigated. Would HSCs remain in a primitive state or differentiate towards a cell lineage in the absence of Notch signaling?

1.4.1.2 Loss-of-function studies are contradictory as to a role of Notch signaling in HSC self-renewal and maintenance

The generation of conditional knock-out mouse models allows the study of the role of Notch signaling in HSC generation and maintenance. Strikingly, while some loss-of-function studies supported a role of Notch in HSC maintenance, others contrastingly showed that Notch signaling has no regulatory function in these cells. As previously mentioned, Kumano and colleagues showed very convincingly that *Notch1*, although redundant for primitive haematopoiesis, is essential for the generation of definitive HSCs from haemogenic endothelial cells (ECs), during embryonic development (Kumano et al., 2003). Even though the numbers of haemogenic ECs are not altered in the *Notch1*$^{-/-}$ embryo, their capacity for haemogenicity is impaired in these mice, where no HSCs were generated in the absence of *Notch1*. Importantly, *in vitro* abrogation of Notch signaling in AGM explants, where CD45^{+} HSCs already existed did not affect HSC generation, suggesting that *Notch1* is indeed essential for the generation of HSCs but not for their maintenance (Kumano et al., 2003). Conversely, *Notch2* is dispensable for

embryonic haematopoiesis (Hadland et al., 2004; Kumano et al., 2003). In the absence of the Notch ligand, *Jagged1* but not *Jagged2,* HSC generation is impaired in the AGM embryonic region (Robert-Moreno et al., 2008). Hence, Notch signaling is essential for the emergence of HSCs in the embryo, where NOTCH1 seems to be the key receptor involved in this process.

Contrastingly, it has been demonstrated, in adult mice through FACS analysis and transplantation assays, involving serial transplantation and limiting dilution transplantation settings through either *dnMAML1*-transduced BM progenitors or validated mouse models, such as the *Rbp-Jk* conditional knockout mouse or the *dnMAML1-GFP* conditional knock-in transgenic, that Notch signaling has no role in HSC self-renewal and maintenance. In steady-state (using the *dnMAML1-GFP* model) or after myeloablation followed by transplantation (using both mouse models), the absence of the Notch signaling pathway did not affect the numbers nor function of HSCs *in vivo* (Maillard et al., 2008). A high recombination efficiency in *dnMAML1-GFP* mice was demonstrated by FACS quantification of green fluorescent protein $(GFP)^+$ cells and Southern blot analysis of the Cre-mediated inactivation of *Rbp-Jk*. In addition, recombination efficiency in both recombined transgenic models was also verified through investigation of the lack of T and marginal zone B cells, as expected from the absence of *Notch1* and *Notch2*, respectively (Maillard et al., 2004; Sambandam et al., 2005). Thus, these studies overall strongly implicate Notch signaling as redundant for HSC maintenance in adult BM. However, these studies are in disagreement with a previous report by Duncan and colleagues where they report that purified HSCs transduced with a dominant negative form of RBP-Jk (DNRBPJ) that binds to NICD but cannot bind

to DNA, when competitively transplanted into recipients are majorly affected in their repopulating capacity, compared to controls. This suggests that Notch signaling is important for self-renewal of HSCs under conditions of great stress (Duncan et al., 2005). The reason for the discrepant results obtained between these two studies is not clear. Nevertheless, the DNRBPJ construct needs further validation for its specificity and side effects on other cellular processes.

Further supporting a redundant role of Notch in HSC maintenance are studies on conditional Rbp-*Jk-, Notch1-* and *Notch2*-deficient mice that failed to show a role for Notch in the HSC compartment, in steady state (Han et al., 2002; Radtke et al., 1999; Saito et al., 2003), although these reports were more directed to evaluate the effects of Notch deficiency in lymphopoiesis and therefore the HSC compartment was not studied in detail. In addition, the number or function of HSCs were unaffected in mice lacking *Jagged1* or both *Jagged1* and *Notch1* (Mancini et al., 2005). In contrast, though redundant for HSC self-renewal in homeostasis, a very recent study using *Notch1*- and *Notch2*-deficient mice, showed that *Notch2* enhances formation of reconstituting HSCs and MPPs shortly and temporarily after transplantation, suggesting a role for this receptor in BM regeneration under conditions of stress (Varnum-Finney et al., 2011).

Importantly, as previously mentioned, Notch ligands are expressed at low levels in the BM. The recently identified negative regulator of Notch signaling Leukaemia/Lymphoma related factor (LRF) in the BM may have a key role in keeping Notch ligands at basal levels implicating an active repression of the Notch signaling pathway in the BM, most likely preventing extrathymic generation of T cells (Maeda et al., 2007).

Thus, the role of Notch signaling in HSC self-renewal and maintenance in adult haematopoiesis remains largely disputed in the field and very stringent and rigorous studies using validated tools are required to resolve the importance of this signaling pathway in HSCs.

1.4.2 Notch in myeloid development in the bone marrow

Notch signaling is indisputably critical for T cell lineage commitment over B cell lineage, in the thymus (Han et al., 2002; Pui et al., 1999; Radtke et al., 2004), therefore playing a key role in lymphopoiesis. Nevertheless, its role in myeloid lineage commitment events in the BM is disputed.

1.4.2.1 Effect of Notch activation in myeloid differentiation

Several overexpression studies in myeloid progenitor cell lines showed that constitutive Notch signaling inhibits differentiation into the myeloid lineages and permits an expansion of undifferentiated cells (Bigas et al., 1998; Carlesso et al., 1999; Milner et al., 1996). Similarly, NICD overexpression studies on primary human and murine cells, demonstrated that stem/progenitor cells retain their self-renewal potential as well as their capacity to form immature colonies and are inhibited from committing towards myeloid lineages (Carlesso et al., 1999; Pui et al., 1999; Stier et al., 2002; Ye et al., 2004). In fact, there are reports showing that mouse progenitor cells overexpressing NICD, preferentially commit towards lymphoid over myeloid lineages (Pui et al., 1999; Stier et al., 2002).

Contrastingly, two studies by Schroeder and colleagues, using the mouse myeloid progenitor cell line 32D or a murine long-term BM cell line, demonstrated that conditional overexpression of NICD enhances granulocytic, myeloid and dendritic cell differentiation while decreasing self-renewal potential (Schroeder and Just, 2000; Schroeder et al., 2003). Accordingly, Tan-Pertel and coworkers reported that constitutively active forms of *Notch1* and *Notch2* receptors support early granulocytopoiesis, however terminal differentiation of these cells was inhibited (Tan-Pertel et al., 2000).

The strong promotion of T cell development in the thymus over other lineages, myeloid included, due to the activation of Notch signaling upon Notch ligand-receptor interactions (Han et al., 2002; Pui et al., 1999; Radtke et al., 1999) and the low levels of Notch ligands in the BM in contrast with very high levels detected in the thymus (Anderson et al., 2001; Bertrand et al., 2000; Parreira et al., 2003), suggest that Notch ligands might also have an important role in lineage commitment events. In fact, *in vitro* culture of human and mouse BM stem and progenitor cells in the presence of DELTA1 ligand resulted in inhibition of myelopoiesis, in a growth factor dependent manner (Ohishi et al., 2002; Ohishi et al., 2001; Varnum-Finney et al., 2003). In one study in particular, inhibition of myelopoiesis was dependent on Notch signaling acting through the GATA-2 transcription factor (de Pooter et al., 2006). Likewise, precursor cells are impaired in differentiating towards the myeloid lineage if cultured in the presence of JAGGED1 (Li et al., 1998; Masuya et al., 2002; Varnum-Finney et al., 1998; Walker et al., 1999).

All together, studies such as the above are contradictory and based on the effect of Notch activation in myeloid differentiation, and are therefore inconclusive regarding the potential physiological role of Notch signalling in myeloid cell commitment and differentiation.

1.4.2.2 Is Notch redundant for myeloid lineage commitment except for the Mk lineage?

Loss-of-function studies investigating in detail the role of Notch signaling in specifying myeloid lineages in the BM have been reported only recently. Although more detailed and complete approaches are required, preliminary data from studies in *Notch1*- and *Rbp-Jk*-deficient mice, applying *in vitro* colony forming assays for the G, M, GM, E, mixed GM/E and B cell lineages as well as FACS staining for mature Mac-1^+ monocytes, $GR1^+$ granulocytes, $Ter119^+$ erythrocytes, and $NK1.1^+$ Nk cells, failed to show a major effect of the absence of Notch signaling in BM lineage commitment (Han et al., 2002; Radtke et al., 1999). Importantly though, the effect of Notch signaling disruption in Mk development was not assessed in these studies.

In contrast, a recent study implicated Notch as an important positive regulator of megakaryopoiesis (Mk) to the detriment of GM and E differentiation (Mercher et al., 2008). In LSK cells pre-stimulated on OP9 stroma expressing *Delta-like 1* (OP9-Dll1), granulocyte-myeloid and erythroid differentiation was suppressed and Mk potential promoted as demonstrated by enhancement of $CD41^+CD42b^+$ Mk cells quantified by FACS and the increase of

Acetylcolinesterase $(AchE)^+$ Mk colonies in *in vitro* CFU assays. Furthermore, when single LT-, ST-HSCs and LMPPs were cultured on OP9 and OP9-Dll1 cells, LT- and ST-HSCs differentiated into Mk in both cultures, contrary to LMPPs, that lacked Mk potential, as reported before (Adolfsson et al., 2005). In addition, LSK cells pre-stimulated on OP9-Dll1 showed upregulation in the expression of Mk related genes, such as *Gata1* and *Fli1*. Of particular significance, these *in vitro* results were further supported by *in vivo* findings. Investigation of Notch target genes such as *Hes1* and *Hes5,* suggested that Notch signaling is activated *in vivo* in MEPs and CMPs, but not GMPs. Furthermore, when analyzing the *dnMAML1-GFP* mouse model, where Notch signaling is disrupted, MEPs were virtually absent while CMPs and GMPs were not affected. Moreover, *dnMAML1*-expressing CMPs showed reduced erythroid and megakaryocytic potential *in vitro* as well as decreased levels of expression of the Notch target genes *Hes1, Hes5* and *Hey1*. Of importance to note, the negative effect on E potential of CMP in the absence of Notch signaling is surprising in the light of Notch activation similarly inhibiting E development in the *in vitro* gain-of-function experiments. Despite this, platelet counts were not affected in mice transplanted with *dnMAML1*-transduced BM cells. However, no competitive transplantation assays were performed and therefore a potential reduction in ability to produce platelets might have been missed.

Thus, loss-of-function studies done to date have been unable to resolve if Notch is fundamental for GM and E lineage fate decisions and development but support a potential role of Notch in specifying Mk lineage commitment, perhaps at the expense of the other myeloid lineages.

1.5 T cell development

T cells play a central role in cell-mediated immunity. These lymphocytes are generated in the thymus, a specialized organ for haematopoiesis where T cell progenitors mature and differentiate (Miller and Osoba, 1967). Histologically, the thymus consists of two symmetrical lobes, each one subdivided into central medulla in the inner part of the organ, followed outward by the cortex that is surrounded by the subcapsular region. The stroma of the thymus is composed by epithelial and dendritic cells (Rodewald, 2008), although developing T-cell progenitors represent the majority of cells existent in the thymus (Miller and Osoba, 1967).

It has been shown that intrathymic and intravenous transfer of thymocytes only support thymopoiesis for a short period of time. Similarly, transfer of BM cells intrathymically only maintains transient thymopoiesis. Conversely, if BM cells are injected intravenously then sustained thymopoiesis is observed (Goldschneider et al., 1986; Scollay et al., 1986). These observations support the notion that the thymus is a non self-renewal organ and therefore requires constant seeding by progenitors from the BM to assure thymopoiesis throughout the life of an individual.

As previously mentioned, progenitors in the embryo migrate from surrounding mesenchyme and blood vessels into the thymic primordium from e11 onwards. In adult life, studies have demonstrated that thymus seeding is not continuous but rather happens in a phasic fashion (Foss et al., 2001). The deceleration in thymus seeding already reflects a phenomenon termed thymus involution that corresponds to a progressive atrophy of thymus activity with age,

characterized by a gradual decrease in thymus cellularity as well as disruption of normal thymus architecture negatively influencing the capability of generating *de novo* T cells. Thus, thymic involution results in a decline in the T cell repertoire concomitant with an accumulation of memory T cells in the organism with aging (Aspinall and Andrew, 2000; Haynes et al., 2000; Steinmann, 1986).

1.5.1 T cell differentiation in the thymus: αβ *versus* γδ T cells

From thymocyte development in the thymus emerge two functionally distinct T cell subpopulations that leave the thymus and migrate to the periphery and are defined according to their T cell receptor (TCR) chain usage; the αβ T cells, which represent the vast majority of mature T cells produced in the thymus and are mainly responsible for the major histocompatibility complex (MHC)-restricted antigen-specific protection against infection, and the non-restricted-MHC γδ T cells, that complement and regulate the activities of αβ T cells, especially in tissues, being also involved in wound healing (Hayday and Pennington, 2007; Hayes and Love, 2007; Kee, 2009). αβ and γδ T cells can be further subcategorized into cells with different functional specializations (Hayday and Pennington, 2007).

1.5.1.1 From DN stages to β-selection events

T cell progenitors coming from the BM enter the thymus at the cortico-medullary junction (CMJ). Once in the thymus, these progenitors mature through four different stages – the CD4/CD8 double-negative (DN) stages – phenotypically

characterized by the fluctuation in the expression of the surface markers CD44 and CD25, which is concomitant with rearrangements of their TCR genes, *TCR α, β* and *γ*, at DN2 and DN3 stages (Figure 1.6).

The most immature DN cells, known as DN1 cells, express CD44 but are negative for the CD25 marker. While DN1 cells migrate outwards through the cortex, CD25 expression is upregulated (DN2) and subsequently CD44 is downregulated (DN3), culminating in the subcapsular zone. Importantly, DN1 and DN2 cells express c-KIT receptor (Godfrey et al., 1993; Godfrey et al., 1992; Porritt et al., 2004). At the DN3 stage, CD44 is downregulated and extensive TCR chain rearrangements occur. T cells expressing a functional γδTCR develop into γδ T cells. Conversely, successful β chain rearrangements and assembled pre-TCR (with pre-Tα and CD3 chains) drive T cells towards the $CD4^+CD8^+$ αβT cell lineage. This process is known as β-selection and corresponds to the differentiation of DN T cells into double positive (DP) T cells (Ceredig and Rolink, 2002). The $CD4^+CD8^+$ DP T cells migrate back through the cortex in the direction of the medulla and undergo selection events that will determine their fate (Figure 1.6).

Through interactions between αβTCR on the thymocyte surface and MHC peptide complexes expressed by the thymic epithelial cells or thymic dendritic cells, DP αβT cells undergo positive or negative selection. If DP T cells recognize MHC class I or class II molecules they receive survival and maturation signals, where expression of $CD4^+$ or $CD8^+$ is lost, respectively – maturing into $CD8^+$ or $CD4^+$ single positive (SP) T cells. If DP T cells cannot recognize MHC molecules or interact too heavily with both MHC class I and class II peptides, then they are instructed to enter apoptosis and are eliminated by thymic macrophages. The

remaining cells can now leave the thymus and migrate to the periphery where they finalize maturing and are functionally activated (Hayday and Pennington, 2007) (Figure 1.6).

Out of the about 50 million thymocytes generated in the thymus of a mouse per day, only 2-3% survive the selection events and join the T-cell repertoire. Importantly, more naïve thymocytes are produced at early ages and the thymus shrinks with age accompanied by a decrease in T cell generation. Therefore, protection of older individuals is maintained by the expansion of peripheral memory T cells (Hayday and Pennington, 2007; Kee, 2009).

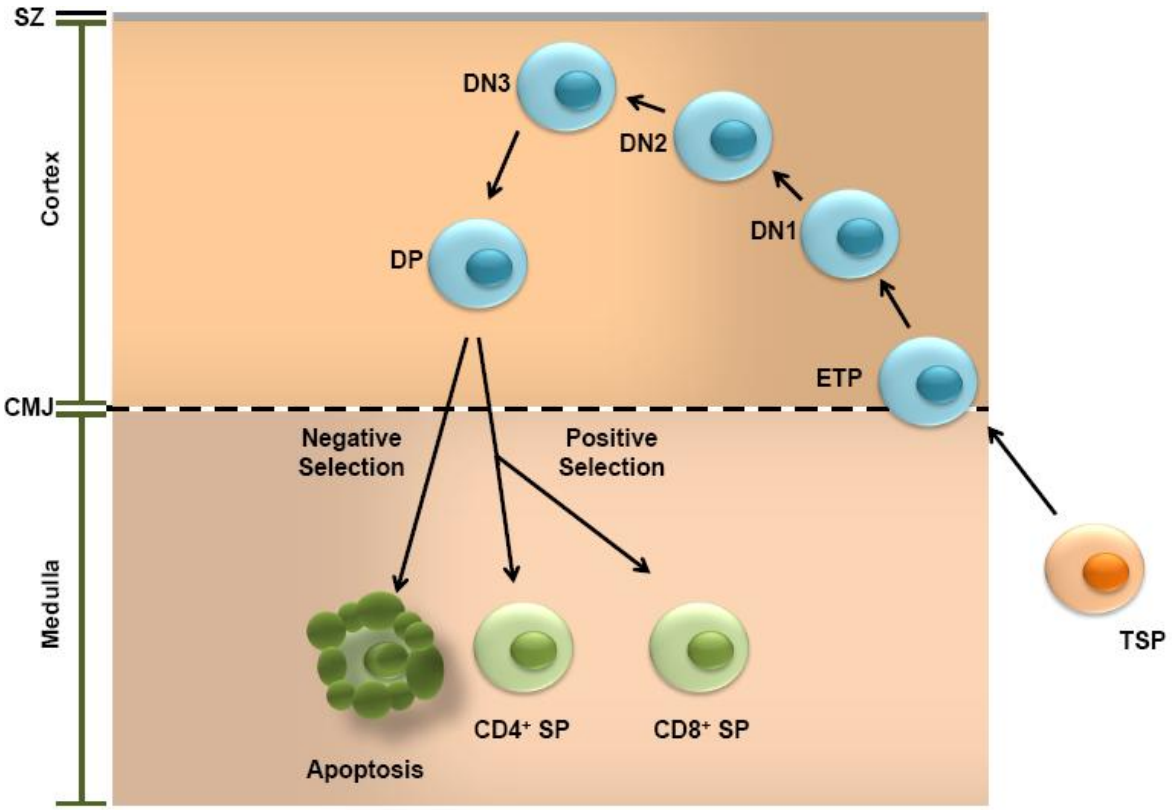


Figure 1.6. Schematic representation of T cell development. SZ: subcapsular zone; CMJ: **c**ortico-medullary junction; TSP: thymus seeding progenitor; ETP: earliest thymus seeding progenitor; DN: double negative; DP: double positive; SP: single positive.

1.5.2 The earliest thymic T-cell progenitor

The DN1 population is a heterogeneous population that contains the most immature progenitor found to date in the thymus. Approximately 0.05% of the DN1 cells express high levels of the cytokine receptor, c-KIT (Allman et al., 2003). This c-KIT^{hi} population within the DN1 subset was further characterized in a study from Allman´s laboratory. DN1 CD44^{+}CD25^{-}c-KIT^{hi} cells were depleted of mature cells, such as DP, SP and immature T cells, γδ T cells, Nk and NKT cells, as well as remaining myeloid and B cells. The majority of these Lin^{-}CD44^{+}CD25^{-}c-KIT^{hi} cells, the so called earliest T-cell progenitors (ETPs), were shown to express, concomitantly with c-KIT, high levels of SCA-1 and low to negative levels of IL7 receptor α (IL7Rα), and represent slightly less than 0.01% of the whole thymus. Functionally, ETPs are very efficient T cell progenitors (Allman et al., 2003). Although very limited, a small fraction of a restricted subset of ETPs which express the CC chemokine receptor CCR9 gives rise to B cells at the single-cell level (Benz and Bleul, 2005). In addition, a very small fraction of FLT3-expressing ETPs retain B cell potential (Sambandam et al., 2005). However, there are studies contesting the existence of B potential within the earliest T cell progenitor population (Balciunaite et al., 2005; Porritt et al., 2004), and anyway it is not clear whether this

limited B cell potential derives from a truly multipotent progenitor or more B cell-restricted progenitors. Nevertheless, it appears that the capability of ETPs to generate B cells is dependent upon the age of the mouse, where B cell potential seems to be higher in younger mice compared to adults (Ceredig et al., 2007). More recently, it has been demonstrated that single ETPs can generate myeloid and T cells *in vitro* and *in vivo.* However, all of these ETPs lacked B cell potential (Bell and Bhandoola, 2008; Wada et al., 2008). ETPs also retain Nk and DC cell potential (Bhandoola et al., 2003). Nevertheless, despite all the efforts trying to functionally characterize the ETPs, the multipotency of these progenitors at the single-cell level remains to be established.

1.5.3 Commitment towards a unipotent T cell progenitor in the thymus

As mentioned before, ETPs at the DN1 stage appear to have combined T, Nk, DC and GM (but probably not MkE, although not investigated) potential. As DN1s progress to the DN2 stage, the capability to generate Nk and DC cells is sustained, but the myeloid potential is substantially reduced in DN2s and completely abolished as cells enter the DN3 stage (Bell and Bhandoola, 2008; Wada et al., 2008). This stage appears to be the first DN stage in which all progenitors have become truly T cell-restricted in their potential, coinciding with TCR rearrangements (Hayday and Pennington, 2007) and also concomitant loss of DC and Nk cell potential (Benz and Bleul, 2005; Schmitt et al., 2004).

1.5.4 Thymus-settling progenitors

1.5.4.1 Bridging between BM and thymus

The thymus is continually seeded with progenitors coming from the BM to guarantee the maintenance of thymopoiesis throughout life. Thymus settling is most likely a very selective and regulated event (Donskoy et al., 2003; Foss et al., 2001), where the availability and capacity of the thymic niches to bear progenitors is the limiting factor (Spangrude and Scollay, 1990). In fact, there are studies showing that the thymus is seeded by progenitors periodically, in waves (Donskoy et al., 2003; Foss et al., 2001).

BM progenitors exit the BM to enter the circulation and eventually seed the thymus (Schwarz and Bhandoola, 2004; Wright et al., 2001). The mobilization of progenitors from the BM to the thymus via the blood requires these cells to become less responsive to chemokines and adhesion molecules that retain them in the BM niches and matrix (Schwarz and Bhandoola, 2006). Concomitantly, BM progenitors should receive signals from cytokines, such as the c-KIT ligand, stem cell factor (SCF), FLT3 ligand (FL), IL-3 and granulocyte-colony stimulating factor (G-CSF) that, although not their main role in progenitors, contribute to their mobilization into and in the blood circulation (Schwarz and Bhandoola, 2006). In addition, chemokines, such as macrophage inflammatory protein (MIP)-α, IL-8 and Groβ, also contribute to the BM progenitors circulating in the blood until they are specifically attracted to the thymus, a process again involving cell adhesion molecules, namely CD44, L-selectin/CD62L, P-selectin/CD62P and PSGL-1, cytokines (Bhandoola et al., 2007; Krueger et al., 2010; Schwarz and Bhandoola,

2006; Schwarz et al., 2007; Zlotoff et al., 2010) as well as chemokine pairs, with special emphasis on the adjacent cystein residue chemokine family receptors, CCR9 and CCR7 (Liu et al., 2006), whereby *Ccr9* and *Ccr7 null* mice have reduced T cell progenitors in the thymus, revealing an important role for these receptors in thymus homing (Benz et al., 2004; Uehara et al., 2002). Interestingly, CCR9 seems to be dependent on FLT3/FL signaling, as $CCR9^{+}$ LMPPs are reduced in mice deficient for *Flt3 receptor* and *Flt3 ligand* (Schwarz et al., 2007).

It has been estimated that approximately 10-100 progenitors enter the thymus per day, in an adult mouse (Schwarz and Bhandoola, 2006; Shortman et al., 1990), which is a very small number and reflects the difficulties of directly identifying the TSP. Therefore, the characterization and comparison, phenotypically and functionally, between BM progenitors and the thymic earliest populations seems to be the most reliable approach towards the identification of the TSP.

1.5.4.2 Candidate BM progenitors of the earliest T cell progenitor

Multiple progenitors with T cell potential in the thymic environment have been identified in the BM. However the identity of the first cell coming from the BM and seeding the thymus as well as whether the thymus is seeded by a single stage of stem or progenitor cells or several progenitors remains unknown.

1.5.4.2.1 The common lymphoid progenitor

The non-renewing capacity and lymphoid-restricted potential of ETPs as suggested by initial studies suggested that its upstream progenitor within the BM should share the same characteristics (Bhandoola et al., 2003). Thus, the CLP arose immediately as the first candidate to settle the thymus and differentiate into mature T cells (Kondo et al., 1997). Accordingly, CLPs are, similarly to ETPs, lymphoid-specific progenitors and lack self-renewal capacity (Allman et al., 2003; Kondo et al., 1997). However, CLPs have a more limited T cell potential and, contrastingly, higher capability to generate B cells than do ETPs (Allman et al., 2003). Nevertheless, one should consider that the environment may modulate the cell potential. In fact, the strong Notch environment in the thymus stimulates T cell development, inhibiting B cell potential, a phenomenon not observed in the BM where Notch signals are much lower than in the thymus (Han et al., 2002; Radtke et al., 1999). Accordingly, CLPs manipulated *in vitro* in the presence of DELTA1 Notch ligand show stronger T cell potential compared to control cultures, where B cell potential is dominant over T cell potential. Thus, one cannot exclude that CLPs could receive instructions while traveling to the thymus or once in the thymic environment that could alter their potential. In addition, both ETPs and CLPs have been recently demonstrated to retain myeloid potential (Bell and Bhandoola, 2008; Ehrlich et al., 2010; Inlay et al., 2009; Wada et al., 2008). Importantly, studies using the Ly6D surface marker have been able to separate CLPs, with B and T cell potential, from B-cell restricted progenitors, within the original CLP population (Inlay et al., 2009).

TSPs travel through the blood to the thymus. However, CLPs, although expressing the important thymus homing molecules, CCR9 and PSGL-1 (Rossi et

al., 2005; Schwarz et al., 2007), have never been detected in the blood or adult thymus (Bhandoola et al., 2007). Nevertheless, entry of CLPs into the thymus via the blood cannot be disregarded, as donor-derived mature T cells are detected in the thymus in a short-period of time after intravenous transfer of CLPs into conditioned mice (Inlay et al., 2009; Serwold et al., 2009).

Phenotypically, CLPs and ETPs share surface markers such as c-KIT and FLT3 receptors. However, CLPs are purified based on the expression of the surface receptor, lL7Rα, while expression of this surface marker by ETPs is still disputed (Allman et al., 2003; Kondo et al., 1997).

All in all, given that CLPs are found to be less potent than ETPs at reconstituting the thymus, ETPs are unlikely to derive from CLPs, but are rather upstream to CLPs and closer to BM LSK progenitors (Allman et al., 2003). Although this hypothesis is still debatable in the field, analysis of mice deficient for *Ikaros*, a lymphoid-restricted zinc finger transcription factor and a master regulator of lymphocyte differentiation, revealed that these mice lack CLPs and therefore B cells but T cell development is intact and ETPs can be detected (Allman et al., 2003; Wang et al., 1996). These results suggest that ETPs develop independently of CLPs, most likely from a progenitor within the LSK compartment in the BM. Contrastingly, recent studies claim that CLPs are in fact the main source of ETPs, where these two populations give similar readouts *in vivo* and *in vitro* when assayed for lymphoid and myeloid potential (Ehrlich et al., 2010).

CLP-2, a $B220^{+}CD19^{-}$preTCRα (pTα) progenitor considered to be downstream of CLP, has been identified within the thymic DN1 compartment of

hCD25 reporter mice controlled by pTα regulatory sequences and has also been suggested as a candidate for the earliest thymus seeding progenitor (Gounari et al., 2002; Martin et al., 2003). However, CLP-2 has been shown to have a more limited T cell potential than ETPs and, although capable of migrating to the thymus after intravenous transfer, these progenitors have never been found in the blood circulation under steady state physiological conditions (Gounari et al., 2002; Martin et al., 2003). Thus, it is unlikely that ETPs derive from CLP-2.

1.5.4.2.2 The circulating T cell progenitor

Another candidate for the TSP is the circulating T cell progenitor (CTP), found in the foetal liver and blood circulation of adult *hCD25* reporter mice and phenotypically characterized as $hCD25^{+}Lin^{-}B220^{-}c\text{-}KIT^{lo}IL7R\alpha^{lo}FLT3^{-/lo}SCA\text{-}1^{+}Thy1.1^{+}$, with high T cell potential, but limited B, Nk and myeloid potential (Krueger and von Boehmer, 2007). Interestingly, the identification of this progenitor supports the hypothesis that, in contrast with the TSP candidates described above, T cell commitment may occur extrathymically (Carlyle and Zuniga-Pflucker, 1998; Krueger and von Boehmer, 2007; Rodewald et al., 1994).

1.5.4.2.3 Progenitors contained in the LSK compartment

ETPs are phenotypically and functionally very similar to BM LSK cells (Allman et al., 2003). LSK is a very heterogeneous population that comprises

HSCs as well as non-renewing multipotent progenitors (Ikuta and Weissman, 1992; Li and Johnson, 1995; Spangrude et al., 1988).

Rescue of conditioned mice with blood leukocytes and exchange of HSCs between parabiose mice, where circulation is conjoined between the two mice, indicates that HSCs are present in the blood (Goodman and Hodgson, 1962; Wright et al., 2001). More recently, FACS analysis of blood from adult mice showed that LSK are indeed present in the blood and are potent T cell progenitors (Schwarz and Bhandoola, 2004).

1.5.4.2.3.1 Haematopoietic stem cells

HSCs are unlikely to be the first progenitors coming from the BM and seeding the thymus themselves because HSCs have never been detected in the thymus (Goldschneider et al., 1986; Mori et al., 2001; Scollay et al., 1986) and cannot rapidly reconstitute the thymus a few weeks after intravenous transfer into unirradiated mice (Schwarz et al., 2007). In addition, although not studied in purified ETPs, erythroid potential has never been reported in the adult thymus (I, 1996; Wu et al., 1991), supporting the view that HSCs cannot reach the thymus. Moreover, HSCs do not express CCR9, which is very important for thymus seeding, although it has been reported that there are CCR9-independent homing processes (Bhandoola et al., 2007; Schwarz et al., 2007).

1.5.4.2.3.2 Lymphoid-primed multipotent progenitor

LMPPs with attenuated myeloid potential, which express the *Rag* gene (Igarashi et al., 2002) and lack VCAM-1 (Lai and Kondo, 2006) but express high levels of FLT3 (Adolfsson et al., 2005), represent a strong candidate for the TSPs. LMPPs give rise to a large number of DP T cells after intravenous transfer, in addition to their B and Nk potential *in vitro* (Adolfsson et al., 2005; Igarashi et al., 2002; Krueger et al., 2010). Furthermore, early lymphoid progenitors (ELPs), which are likely to largely overlap with LMPPs, are found in the blood circulation (Perry et al., 2006) and are the first MPPs expressing CCR9, an important receptor for thymic settling (Schwarz et al., 2007). Notably, B cell potential in the ETP population is greatly reduced compared to LMPPs (Allman et al., 2003).

Additionally, ETPs are at a "crossroads" of lineage options and therefore, besides expressing master T lineage genes such as *Gata3*, *Cd25* or *preTα*, these progenitors sustain B cell gene expression, namely of e*arly B cell factor (Ebf)* as well as myeloid, DC and Nk cell genes, including *PU.1, CCAAT/Enhancer-binding protein-alpha (Cebpα)* and *Inhibitor of DNA binding 2* (*Id2)*, keeping open the non-T cell lineage options at this stage of T cell development (Lai and Kondo, 2007; Rothenberg et al., 2008). Accordingly, GM lineage associated genes are also detected in LMPPs reflecting their retained myeloid potential (Mansson et al., 2007). Conversely, E and Mk genes are dramatically downregulated in LMPP progenitors, in agreement with their lack of MkE potential *in vivo and in vitro* (Adolfsson et al., 2005; Luc et al., 2008a; Mansson et al., 2007). Data from my laboratory show that ETPs have functionally lost their MkE potential and that at the transcriptional level, MkE related genes are expressed at very low levels. Thus, ETP and LMPP gene expression signatures must be reasonably comparable,

further arguing towards a progenitor-successor relationship. Nevertheless, an LMPP-like cell remains to be found in the thymus.

1.5.4.3 Concluding remarks

Although several BM progenitors have been suggested as possible candidates for TSPs, their identity remains to be resolved. In addition, it should be considered whether thymus is settled by multiple BM progenitors, rather than a single progenitor that may commit into T cell progenitors through different routes in the thymus. Furthermore, resolving this question would also bring insight into the origin of ETPs and their relationship with phenotypically and functionally identical progenitors identified in the BM.

1.5.4.4 Intrathymic or extrathymic T cell commitment – an important note

Whether T cell commitment occurs exclusively intrathymically or some progenitors commit towards T cell lineage before seeding the thymus remains unresolved in the field. The existence of thymic B/M and B/T progenitors (Bell and Bhandoola, 2008; Benz and Bleul, 2005; Wada et al., 2008) suggests that progenitors lock into the T cell lineage once they seed the thymus and receive Notch signaling instructions. However, the identification of single lineage T cell progenitors in the blood (Krueger and von Boehmer, 2007) and BM (Maillard et al., 2006) argue against exclusive intrathymic T cell commitment. It can also not be

excluded that rare T cell restricted progenitors in the BM and peripheral blood might derive from thymic progenitors. Nevertheless, new methods now allow one to evaluate the lineage multipotency of a single cell which is a powerful tool helping to resolve the existence of a multipotent progenitor in the thymus and thus determine whether lineage commitment of a multipotent progenitor from the BM into a unipotent T cell progenitor occurs within the thymus. Even so, intrathymic and extrathymic T cell commitment are not necessarily mutually exclusive and may both occur, considering that the thymus may be settled by multiple progenitors that follow different lineage restriction paths, before and after reaching the thymus, towards a T cell progenitor (Petrie and Kincade, 2005).

1.6 Notch signaling in T cell development

The role of Notch signaling in T cell development is its best known role in haematopoiesis (Radtke et al., 2010). Nevertheless, the first cell receiving Notch signals and where these signals are first delivered remains unknown.

1.6.1 Notch signaling is a positive regulator of T cell development in the thymus

Notch1 and *Rbp-Jk* deficiency results in an arrest in T cell development at the DN1 stage with concomitant detection of B cells within the thymus (Benz et al., 2008; Han et al., 2002; Radtke et al., 1999). Conversely, overexpression of the *Notch1* intracellular domain or *Dll4* ligand in BM progenitors leads to ectopic

generation of T cells and a block in B cell development in the BM (Dorsch et al., 2002; Pui et al., 1999; Yan et al., 2001), with the mice eventually developing leukemia (Pear et al., 1996; Pui et al., 1999). Similarly, ectopic expression of *Hes1* and *Hes5* in the BM inhibits B cell generation (Kawamata et al., 2002).

More recently, Maeda *et al* showed that mice lacking *Lrf*, an inhibitor in the Notch signaling pathway, are incapable of generating B cells in the BM and, similarly to what has been observed in mice overexpressing *NICD*, ectopic BM T cells are detected in these mice. These observations are directly related to an effect on Notch signaling, as demonstrated by an upregulation of Notch target genes (Maeda et al., 2007). Thus, LRF negatively regulates Notch signaling in the BM, allowing B cell development to take place. Also, LRF inhibitory action is not observed in the thymus, most likely due to the fact that the Notch ligand expression in this organ is very abundant, overcoming the negative effect of LRF inhibitory function (Maeda et al., 2007). Alternatively, LRF activity might be somehow repressed in the thymus, allowing the development of T cells to occur. Nevertheless, these hypotheses remain to be confirmed.

Likewise, overexpression of other identified Notch signaling inhibitors, such as *Fringe, Deltex, Nrarp* as well as the engineered dominant-negative form of *MAML1* (*dnMAMl1*), also results in a block in T cell development in the thymus (Radtke et al., 2010). Surprisingly, overexpression of *Numb*, yet another negative regulator of Notch, has no effect on T cell development (French et al., 2002).

Hence, all in all, the studies above strongly support the idea that Notch signaling directs progenitors towards the T cell lineage, inhibiting B (Han et al.,

2002; Radtke et al., 1999), myeloid (Wada et al., 2008) Nk and dendritic cell (Feyerabend et al., 2009) differentiation.

1.6.2 NOTCH1 is the key Notch receptor regulating T cell development

In contrast to the striking T cell phenotype in *Rbp-Jk* and *Notch1 null* mice (Han et al., 2002; Radtke et al., 1999), *Notch2* conditional knock-out mice show no defects in T cell generation (Saito et al., 2003). Moreover, no defects at all in haematopoiesis have been reported as of today in *Notch3*-deficient mice (Krebs et al., 2003). All together, these data not only show that NOTCH1 is the most important receptor for T cell development but also that it can compensate for the absence of the other receptors in T cell lineage commitment events in the thymus. Important to note, the effect of NOTCH1 in T cell development in the absence of all three other Notch receptors has never been investigated and therefore a joint role of NOTCH1 with another Notch receptor cannot be excluded.

1.6.3 Notch ligands intervening in thymic T cell development

Notch ligands are widely expressed in BM and thymic stromal cells (Felli et al., 1999). *In vitro* studies using cell lines ectopically expressing the Notch ligands *Dll1* and *Dll4* (mostly the OP9 cell line), early on suggested that these two ligands are very efficient in activating Notch signaling (Hozumi et al., 2004; Schmitt and Zuniga-Pflucker, 2002). In contrast, T cell development is not affected by the absence of *Dll1 in vivo*, while mice where *Dll4* is conditionally deleted from TECs

are unable to produce T cells and generate ectopic B cells in the thymus. Furthermore, DLL4 binds with higher affinity to NOTCH1 receptor than does DLL1 (Hozumi et al., 2008a; Hozumi et al., 2004; Koch et al., 2008). These data are consistent with the gene expression pattern of these two ligands in TECs, where *Dll4* but not *Dll1* is expressed at high levels in the thymus (Hozumi et al., 2008a; Koch et al., 2008). Together, the above data suggest that DLL4 is the main physiological Notch ligand involved in T cell development in the thymus.

1.6.4 Notch is essential for the establishment of ETPs in the thymus

ETPs are included in c-KIT^{hi} expressing DN1 thymic progenitors (Allman et al., 2003). First studies on *Notch1*- (Radtke et al., 1999) and *Rbp-Jk*-deficient mice (Han et al., 2002) showed an expansion of DN1 cells, however how Notch signaling disruption affected ETPs was not investigated. Recent studies suggest that Notch signaling is important for T cell lymphopoiesis upstream to ETPs (Sambandam et al., 2005; Tan et al., 2005). Sambandam *et al* showed that mice transplanted with *dnMAML1*-transduced HSCs virtually lack donor-derived ETPs or DN2s, indicating that Notch signaling is required upstream to or at the same stage as ETPs (Sambandam et al., 2005). Yet another study demonstrates that mice overexpressing the Notch signaling inhibitor *Lunatic Fringe* in the thymus generate less ETPs and DN2s compared to wild type controls (Tan et al., 2005).

Moreover, along with *Notch1 receptor,* it has been demonstrated that the levels of expression of the Notch target genes *Deltex1, Hes1* and *Cd25* increase from HSCs, where levels are extremely low, to LSKs and MPPs, and are further

upregulated in ETPs (Lai and Kondo, 2007; Sambandam et al., 2005). Furthermore, *Deltex1* and *Hes1* expression increases from ETPFlt3^{+} to ETPFlt3^{lo}, indicating that ETPFlt3^{lo} may be downstream to ETPFlt3^{+}. The same genes are still detectable at similar or even higher levels of expression in DN2 and DN3 cells (Sambandam et al., 2005). Thus, these molecular studies suggest an activation of Notch signaling in ETPs (Lai and Kondo, 2007; Sambandam et al., 2005), further corroborating the *in vivo* studies described above. However, and very importantly, these studies do not prove whether this gene activation is indeed Notch signaling dependent.

In conclusion, studies to date implicate Notch signaling, and in particular NOTCH1, in the establishment of ETPs from a BM progenitor.

1.6.5 Is Notch signaling active before TSPs enter the thymus?

Notch signaling has been shown to be stronger in the thymus, reflected by the high number of Notch ligands and receptors in the thymic stroma and T cell progenitor cells, respectively as well as by an upregulation in the levels of expression of Notch signaling related factors, namely Notch target genes in haematopoietic cells residing in the thymus. However, an extrathymic activity of Notch cannot be disregarded, where Notch would play a role in instructing a BM progenitor to leave the BM and seed the thymus as well as restricting its lineage potential towards a T cell fate.

It has been demonstrated that foetal lymphoid progenitors show Notch activation only after entry into the thymic epithelial environment (Harman et al.,

2003). Furthermore, it is not yet resolved as to whether Notch is implicated in cell lineage commitment events outside the thymus. MPPs and LMPPs are not affected by disruption of Notch signaling (Maillard et al., 2008). In addition, BM LSK progenitors are still detected in the blood in the absence of Notch signaling, indicating, further, a lack of involvement of Notch in BM progenitors´ mobilization via blood, eventually from the BM to the thymus (Sambandam et al., 2005). Thus, the data above supports the possibility that Notch signaling has no role in cell commitment events in an extrathymic environment.

It is important to stress that a low intensity of Notch signals is required for inhibition of B cell potential, however high levels of Notch signaling are necessary to allow T cell commitment (Schmitt et al., 2004; Tan et al., 2005) . This is in agreement with low Notch signaling in the BM but intense in the thymus, suggesting that the high levels of Notch signaling in this organ allow the ideal conditions for T cell commitment to take place.

Thus, it remains to be determined whether Notch signaling is already activated on TSPs in the BM and/or blood, before their entry into the thymus, and how this activation influences lineage commitment in BM progenitors.

1.6.6 Role of Notch in T cell lineage restriction from multipotent progenitors

Notch1-deficient mice show an impairment in the establishment of ETPs in the thymus (Benz et al., 2008), however, although very rare, a few DN2 cells can be detected, as well as DP and SP T cells (Han et al., 2002; Radtke et al., 1999).

Nevertheless, it is not clear from these studies whether *Notch1* is important for the DN1-DN2 transition, although it seems to be involved in the upregulation of CD25 and concomitant downregulation of FLT3 protein expression at the DN2 stage (Sambandam et al., 2005). Furthermore, it remains to be studied whether the DN2-DN3 transition is Notch signaling dependent. Conversely, it has been shown that Notch signaling is important for the transition of the DN3 to the DN4 stage, although CD4/CD8 T cell development is not affected by the absence of Notch (Tanigaki et al., 2004; Wolfer et al., 2001). It is important to note that Notch is required for DN3 β-selection and assures survival of the selected thymocytes (Ciofani and Zuniga-Pflucker, 2005; Wolfer et al., 2002).

Thus, although Notch signaling is important at several stages of T cell maturation events in the thymus, it remains unclear whether it plays a role in the loss of alternative lineage fates that occurs from DN1 up to the DN3 stage, where full T cell commitment occurs. Accordingly, several studies have suggested that Notch promotes T cell development by inhibiting Nk, DC, myeloid and B cell production and that myeloid potential is lost after loss of B cell potential (Bell and Bhandoola, 2008; Feyerabend et al., 2009; Han et al., 2002; Laiosa et al., 2006; Radtke et al., 1999; Wada et al., 2008). However, the stepwise lineage restriction of the TSP towards a fully committed T cell progenitor in the thymus remains unknown as well as the role of Notch in these restriction events (Figure 1.7).

1.7 The role of GATA3 in regulation of early haematopoiesis and T cell development

The transcription factor GATA-binding protein 3 (GATA3) is a member of the GATA family. In mammals, the GATA family is composed of six GATA members, GATA1-6, which can be further divided into two different groups according to the tissues where these transcription factors are expressed. The haematopoietic group, that comprises GATA1-3 and the endodermal group, composed by GATA4-6. All these factors share a highly conserved DNA-binding domain composed by two zinc-finger motifs (DNA binding domains where cysteine and histidine residues are coordinated by zinc atoms) in the carboxyl-terminal region that recognize the DNA consensus sequence WGATAR, in which W denotes A or T, and R denotes A or G (Ho et al., 2009; Ko and Engel, 1993; Merika and Orkin, 1993).

Gata3 null mice die around embryonic day 11, due to noradrenaline deficiency that leads to cardiac dysfunction (Lim et al., 2000; Moriguchi et al., 2006; Pandolfi et al., 1995). *Gata3* is expressed in tissues such as the central nervous system, skin, breast and the haematopoietic system among others, in which conditional gene-targeting approaches have demonstrated GATA3 to play important roles (Ho et al., 2009).

1.7.1. GATA3 and the haematopoietic system

In the haematopoietic system, *GATA3* is expressed in both foetal and adult haematopoiesis (Bertrand et al., 2005; Hosoya et al., 2009). In the embryo, *Gata3* is detected in $CD45^{-/lo}$c-$KIT^{+}CD31^{+}AA4.1^{+}$ mouse HSCs (Bertrand et al., 2005) as

well as in ETPs (Hosoya et al., 2009). In adult mice, *Gata3* is mainly expressed in maturing as well as mature T cells, in Nk cells and CD1d-restricted NkT cells (Akashi et al., 2000; Ho et al., 2009; Sambandam et al., 2005). Although involved in several stages of T cell development, GATA3 has been demonstrated to be the master regulator of T helper 2 (Th2)-cell differentiation (Ho et al., 2009; Zheng and Flavell, 1997; Zhu et al., 2004).

1.7.1.1 GATA3 and BM haematopoiesis

Gata3 is expressed in HSCs, CLPs and T cell progenitors, but absent in MEP and GMP myeloid progenitors and barely detected in CMP subsets (Akashi et al., 2000; Benveniste et al., 2010; Lai and Kondo, 2007; Sambandam et al., 2005; Tydell et al., 2007).

Moreover, the expression pattern of *Gata3* in HSCs has been debatable. *Gata3* is expressed at low levels in the LSKIL7Rα^{-} HSCs (Akashi et al., 2000) and downregulated from long-term to short-term reconstituting HSCs. In contrast, a different study rather suggests increasing levels of *Gata3* from HSCs, defined as LSKCD27^{-}, to LSKCD27^{+} multipotent progenitors (Tydell et al., 2007), a discrepancy that can be explained by the different phenotypes investigated. Moreover, important genes involved in HSC maintenance, such as *Cited2, Mpl and c-kit* (Chen et al., 2007; Kent et al., 2008; Kranc et al., 2009; Qian et al., 2007) contain GATA3 binding sites (Zhong et al., 2005). Taken together, gene expression studies suggest that *Gata3* may have a regulatory function in HSCs. However, a

functional role of GATA3 in HSCs has not been extensively investigated to date and remains to be established.

In an early study, *Gata3*-deficient embryonic stem cells (ESC) were injected into blastocysts and multilineage regeneration was assessed (Ting et al., 1996). As expected, no T cells were generated in the chimaeric mice, consistent with the crucial role of *Gata3* in T cell development (Ho et al., 2009). However, myeloid and B cell lineages reconstituted normally (Ting et al., 1996). Similarly, foetal liver *Gata3*-deficient and wild type cells transplanted into myeloablated recipients contributed at similar levels for short-term regeneration of myeloid and B cell lineages (Hosoya et al., 2009). Taken together, these studies indicate that *Gata3* is dispensable for the maintenance of HSCs. In contrast, GATA2 has been shown to be critical for proliferation and survival of HSCs (Ling et al., 2004; Rodrigues et al., 2005; Tsai et al., 1994; Tsai and Orkin, 1997). Therefore, it should be taken into consideration that *Gata2* may be compensating for the absence of *Gata3* in the maintenance of HSCs. Accordingly, redundancy between GATA factors has been shown in the haematopoietic system (Chen and Zhang, 2001). Finally, studies described above failed to investigate a role of *Gata3* in regulating the self-renewal capability of HSCs, a fundamental requirement to establish whether *Gata3* is important or not in the stem cell compartment in the BM.

1.7.1.2 GATA3 in T cell development

Gata3-deficent mice do not develop a rudimentary thymus (Lim et al., 2000; Moriguchi et al., 2006), implicating *Gata3* as an important factor involved in T cell

development. To overcome embryonic lethally of lost *Gata3* and study its role in T lymphopoiesis, blastocysts injected with *Gata3*-deficient ESCs were transferred into *Rag-2 null* mice (Ting et al., 1996). Chimaeric mice were unable to generate T cells, while the other lymphoid lineages were unaffected (Ting et al., 1996), strongly implicating a role of *Gata3* in T cell generation. Nevertheless, these studies could not demonstrate at which stages of T cell development *Gata3* is required.

1.7.1.2.1 Gata3 is important for the early stages of T cell development

Gata3 mRNA levels increase from $VCAM^+$ MPP to the more lymphoid-primed $VCAM^-CCR9^+$ MPP and even further in the ETPs (Lai and Kondo, 2007; Sambandam et al., 2005), which could implicate an involvement of GATA3 in lymphoid priming. Moreover, *Gata3* suppression or overexpression in fetal liver progenitors resulted in loss or arrested cell expansion of $Thy1^+$ T cells *in vitro,* respectively (Anderson et al., 2002; Chen and Zhang, 2001; Hattori et al., 1996a; Hozumi et al., 2008a; Taghon et al., 2001; Taghon et al., 2007). Taken together, these studies suggest that GATA3 may have a role in T cell development from early stages, namely lymphoid priming in the BM and a more prominent role in T cell commitment and maturation in the thymus. However further investigation is required (Figure 1.7).

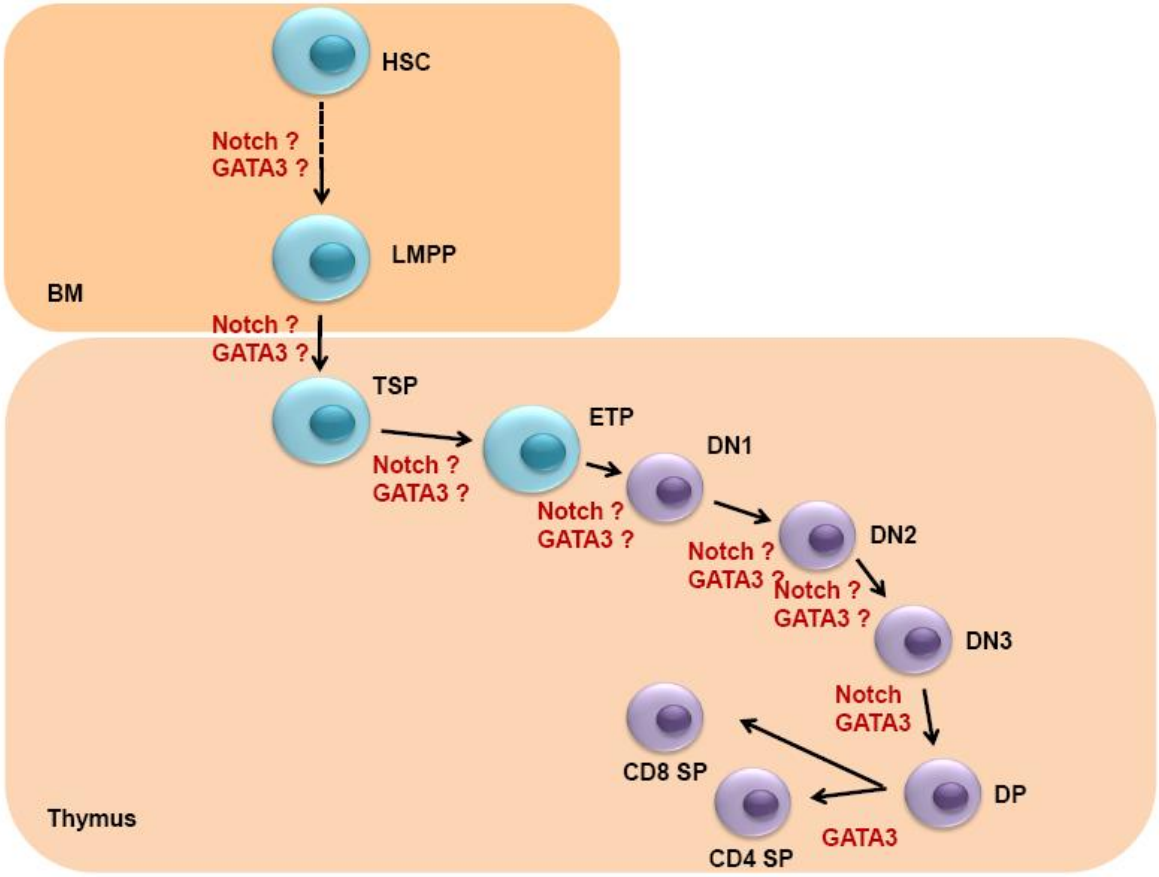


Figure 1.7. Role of Notch and GATA3 in T cell development. Red text shows the role of Notch and GATA3 in T cell lineage specification. Question marks indicate cell lineage specification events in which the role of Notch and GATA3 is not yet fully understood. BM: bone marrow, HSC: haematopoietic stem cell, LMPP: lymphoid-primed multipotent progenitor, TSP: thymus seeding progenitor; ETP: earliest T-cell progenitor, DN: double-negative, DP: double-positive, SP: single-positive. Dashed arrow from HSC to LMPP indicates that there are intermediate multipotent progenitor stages between these two cell populations.

1.7.1.2.2 GATA3 *in later stages of T cell development*

Levels of *Gata3* are upregulated from DN1 through DN3 stages, and downregulated in DN3-DN4, when β-selection occurs (Tydell et al., 2007).

Mice deficient for *Gata3* at the DP and SP stages of development have been generated. In these mice, *Gata3*-deficient DN3 cells were more prone to fail β-selection and therefore undergo apoptosis. However the mechanism by which GATA3 is involved in this checkpoint event remains unknown (Pai et al., 2003). Some studies implicate GATA3 to be involved in the transcriptional regulation of TCR chains as well as the CD8α molecule (Henderson et al., 1994; Ho et al., 1991; Landry et al., 1993). In addition, GATA3 is also involved in the generation and maintenance of CD4 thymocytes (Pai et al., 2003).

After leaving the thymus and colonizing secondary lymphoid organs, $CD4^+$ T cells further differentiate into effector T cells capable of producing cytokines conferring immunity against microorganisms. Depending on the cytokines they produce, effector T cells can be categorized as Th1, Th2, Th17 and regulatory T cells (Ho et al., 2009). Regulation of Th2 cells by GATA3 has been extensively studied and is its best known role in haematopoiesis (Ho et al., 2009; Zheng and Flavell, 1997; Zhu et al., 2004). In fact, GATA3 regulates the differentiation of $CD4^+$ T cells into Th2 cells, which can be dependent or independent of the STAT6 pathway (Ho et al., 2009; Zheng and Flavell, 1997; Zhu et al., 2004).

1.7.1.3 Concluding remarks

Although very well established in the later stages of differentiation, the role of GATA3 in the HSC maintenance as well as establishment of ETPs and in early T

cell commitment events requires further investigation. In fact, it remains unknown, just as in the case of Notch, which are the first cells in which *Gata3* expression occurs and where this factor starts being expressed: is it in the BM or only in the thymus? Is *Gata3* involved in TSP homing to the thymus or is it only important once the TSP has settled into the thymus?

1.7.2 GATA3 and Notch – an alliance in the name of T cell development?

As described above, both GATA3 and Notch signaling are important for T cell development (Ho et al., 2009; Radtke et al., 2010). In addition, gene expression levels of *Gata3*, similarly to *Notch1*, increase from HSCs to thymic populations (Akashi et al., 2000; Hosoya et al., 2009; Lai and Kondo, 2007; Sambandam et al., 2005; Tydell et al., 2007; Zhong et al., 2005). These observations suggest that GATA3 may be regulated by Notch. In fact, GATA3 has been demonstrated to be a direct transcriptional Notch target in Th2 cell differentiation, where RBP-Jk binds to the upstream promoter of *Gata3* (Amsen et al., 2007; Fang et al., 2007). Furthermore, culture of BM uncommitted progenitors on the Notch ligand DELTA1 expressing OP9 cell line are driven towards a T cell fate which is accompanied by an upregulation of *Gata3*. This upregulation is not observed when Notch ligands are removed, indicating that Notch and *Gata3* may interact (Hoflinger et al., 2004; Schmitt and Zuniga-Pflucker, 2002; Taghon et al., 2005). Nevertheless, the mechanism by which Notch and GATA3 interact in the processes of T cell commitment in the thymus remains to be elucidated.

One question that arises from the observations above is whether GATA3 is regulated by Notch signaling or whether enforced expression of *Gata3* could bypass the requirement for Notch signals. However, technical limitations have made it difficult to address these questions. Enforced expression of *Gata3* reduces the cell yield and increases apoptosis, as shown by several studies in human and mouse BM and thymic cells transduced with *Gata3* and cultured for differentiation *in vitro* (Chen and Zhang, 2001; Taghon et al., 2001). If Notch promotes T cell commitment upregulating *Gata3* expression, one would expect that ectopic expression of this factor could potentiate T cell development in the absence of Notch signaling. However, growth of LSK, LSKCD27^{+}Flt3^{+}Il7Rα^{+} lymphoid progenitors and thymic DN subsets was inhibited by *Gata3* overexpression in the presence of DLL1 ligand *in vitro* (Taghon et al., 2007). Moreover, a block in T cell development at the DN1-DN2 stages was observed when DN-containing thymocytes were cultured on Notch ligands (Taghon et al., 2007). On the other hand, lymphoid progenitors cultured in the absence of Notch ligands but overexpressing *Gata3* could not read out for T cells *in vitro.* Taken together, these studies suggest that in the presence of Notch signaling, elevated levels of *Gata3* block cells at an early stage of T cell development, the DN1-DN2 stage, inhibiting them from further progress and committing them towards the T cell lineage. It is worthy of note that the absence of *Gata3* was also detrimental for survival of haematopoietic progenitors from foetal liver cells, even though Notch activation was detected (Hozumi et al., 2008b). All in all, the studies above indicate that, in the absence of Notch signaling, GATA3 is not sufficient to promote T cell development and that the “dose” of *Gata3* in the cell needs to be very well

constrained, as both overexpression and lack of *Gata3* seem to be toxic for survival and development of T cells.

In conclusion, the role of GATA3 in early T cell commitment remains unresolved. Whether it is important for homing to the thymus by BM progenitors and also whether it acts on TSPs to drive T cell commitment is also unknown. Furthermore, the mutual roles of Notch and GATA3 in early T cell commitment events remain to be clarified.

1.8 Aims of this study

Determining the role of Notch signaling and GATA3 in the BM and thymus is of major importance to understanding haematopoietic cell maintenance, proliferation and cell lineage commitment in the mouse. The aims of this study are as follows:

- To resolve the role of Notch, if any, in HSC maintenance and myeloid lineage commitment in the BM and investigate how the transcription factor RBP-Jk regulates Notch target gene activation;

- To study the role of Notch signaling and GATA3 in early T cell commitment, namely the establishment of ETPs in the thymus;

- To analyze *Gata3*-deficient BM with the aim of understanding whether GATA3 contributes to HSC maintenance and self-renewal.

2

Canonical Notch signaling is dispensable for myeloerythropoiesis: suppression of Notch target genes by RBP-Jk

2.1 Introduction

NOTCH1 is crucial for the emergence of definitive HSCs in the embryo (Kumano et al., 2003) and is an important mediator of T cell commitment in the thymus (Radtke et al., 2010). In contrast, the role of Notch signaling in cell maintenance and lineage commitment in the BM remains much disputed (Maillard et al., 2003; Radtke et al., 2010). Gain-of-function studies suggest Notch to be essential for the expansion of adult HSCs, hindering them in differentiating towards a lineage restricted progenitor (Calvi et al., 2003; Kunisato et al., 2003; Stier et al., 2002; Varnum-Finney et al., 1998; Varnum-Finney et al., 2000). In contrast, most lack-of-function studies, using mouse models such as the *Rbp-Jk, Notch1, Jagged1* and double *Jagged1-Notch1* conditional knock-out mice, suggest that this signaling pathway is dispensable for the maintenance of adult HSCs (Han et al., 2002; Maillard et al., 2008; Mancini et al., 2005; Radtke et al., 1999; Tanigaki et al., 2002), while other studies rather implicate a regulatory role of Notch in HSCs, in the BM (Duncan et al., 2005; Varnum-Finney et al., 2011).

Similarly, the role of Notch signaling in lineage fate decisions downstream to HSCs in the BM remains unresolved. In fact, although some gain-of-function studies support a positive effect of Notch in myelopoiesis (Schroeder and Just, 2000; Schroeder et al., 2003), other studies suggest Notch to be a suppressor of myeloid differentiation (Bigas et al., 1998; de Pooter et al., 2006; Masuya et al., 2002; Stier et al., 2002). Moreover, whereas early loss-of-function studies in *Notch1*- and *Rbp-Jk*-deficient mice, focusing primarily on the HSC and T cell phenotypes, suggested that myeloid lineages are not affected by the absence of

Notch signaling, a recent and more detailed study implicated Notch as a positive regulator of Mk cell development from HSCs (Mercher et al., 2008). An explanation for the discrepancy between these studies is not clear. However, it may in part be related to the Cre systems used to induce gene deletion. In fact, it has been recently shown that Cre-recombination induced by poly(I:C) treatment triggers immunological responses with negative effects on HSCs and haematopoiesis (Essers et al., 2009). Either way, more stringent studies are required to resolve whether Notch signaling plays any role in HSC maintenance as well as lineage commitment events, namely megakaryopoiesis, in the BM.

Notch signals coming from all four Notch receptors on the cell surface converge onto the same DNA-binding transcription factor in the nucleus, the RBP-Jk, triggering transcriptional gene activation (Kopan and Ilagan, 2009). In *Drosophila*, transcriptional expression of Notch target genes in the E(spl) complex increases in the absence of the CSL protein, Su(H) (homolog of RBP-Jk in flies) (Bailey and Posakony, 1995). In addition, a more recent study in mouse embryonic fibroblasts showed that the Notch target gene, *Hey1,* is derepressed in the absence of *CtBP*, a corepressor recruited by the Rbp-Jk/SHARP complex to silence Notch target genes (Oswald et al., 2005). These studies suggest that CSL acts as an active transcriptional repressor in the absence of active Notch signalling. However, the activated or repressed state of Notch target genes by RBP-Jk in primary mammalian cells *in vivo* has never been investigated.

When NICD reaches the cell nucleus it binds to the DNA-bound RBP-Jk triggering a displacement of the RBP-Jk associated co-repressor complex by a co-activator complex leading to transcriptional activation of Notch target genes such as *Hes1, Hes5* and *Nrarp* (Bray, 2006). However, although generally accepted, this mechanism of target gene activation has been challenged. In *Drosophila*, Su(H) is instructed to bind to DNA by the NICD (Krejci and Bray, 2007). Thus, it is important to establish the role of RBP-Jk in the regulation of Notch target genes and whether their activation state is dependent upon Notch signaling.

2.2 Results

2.2.1 Steady state maintenance of HSCs and myeloid progenitors in the BM is independent of Notch signaling

In order to assess how Notch signaling affects numbers and function of HSCs and myeloid progenitors, mice carrying the *Rbp-Jk* gene harboring *loxP* sites flanking the sixth and seventh exons, which encode part of the DNA-binding domain crucial for the RBP-Jk function (Han et al., 2002), were used. *Rbp-Jk* mice were crossed with to *Mx1-Cre* transgenic mice and Cre recombinase expression was induced by treating *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ and *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Mx1-Cre*$^{tg/+}$ control mice with poly(I:C) four times, every other day and analysed 4-5 weeks after the last poly(I:C) injection. To further rule out any possibility of non specific effects from the Cre recombinase induction that could have been behind the conflicting results as to the role of Notch in HSCs and myelopoiesis, we also

used *Rbp-Jk*$^{fl/fl}$ mice bred against *Vav-Cre* expressing mice, to obtain a *Vav-Cre*-mediated gene inactivation that occurs specifically in the haematopoietic and endothelial lineages (Georgiades et al., 2002) and starts around day e10, in the embryo (Stadtfeld and Graf, 2005), immediately after HSCs have been generated (Chen et al., 2009). Moreover, *Vav-Cre* is constitutively activated (Georgiades et al., 2002) thus avoiding all the resulting negative side effects from the poly(I:C)-mediated induction of Cre-recombinase (Essers et al., 2009; Furuta and Behringer, 2005; Kuhn et al., 1995).

First, the efficiency of Cre-recombination under the *Vav* promoter was assessed. *Rosa26*$^{YFP/+}$ mice were crossed to *Vav-Cre*$^{tg/+}$ transgenic mice and yellow fluorescent protein (YFP) expression was quantified by FACS in the BM. In agreement with previous reports (Stadtfeld and Graf, 2005), BM cells recombined very efficiently, as demonstrated by the YFP expression levels in unfractionated BM as well as LSK progenitors (Figure 2.1). Recombination in *Vav-Cre* transgenics was detected in all haematopoietic tissues, at very high levels of efficiency (Georgiades et al., 2002). Although not investigated here, it has been previously shown that *Vav-Cre*-mediated recombination is very rare in other somatic cell types outside the haematopoietic and endothelial systems (de Boer et al., 2003; Georgiades et al., 2002; Stadtfeld and Graf, 2005).

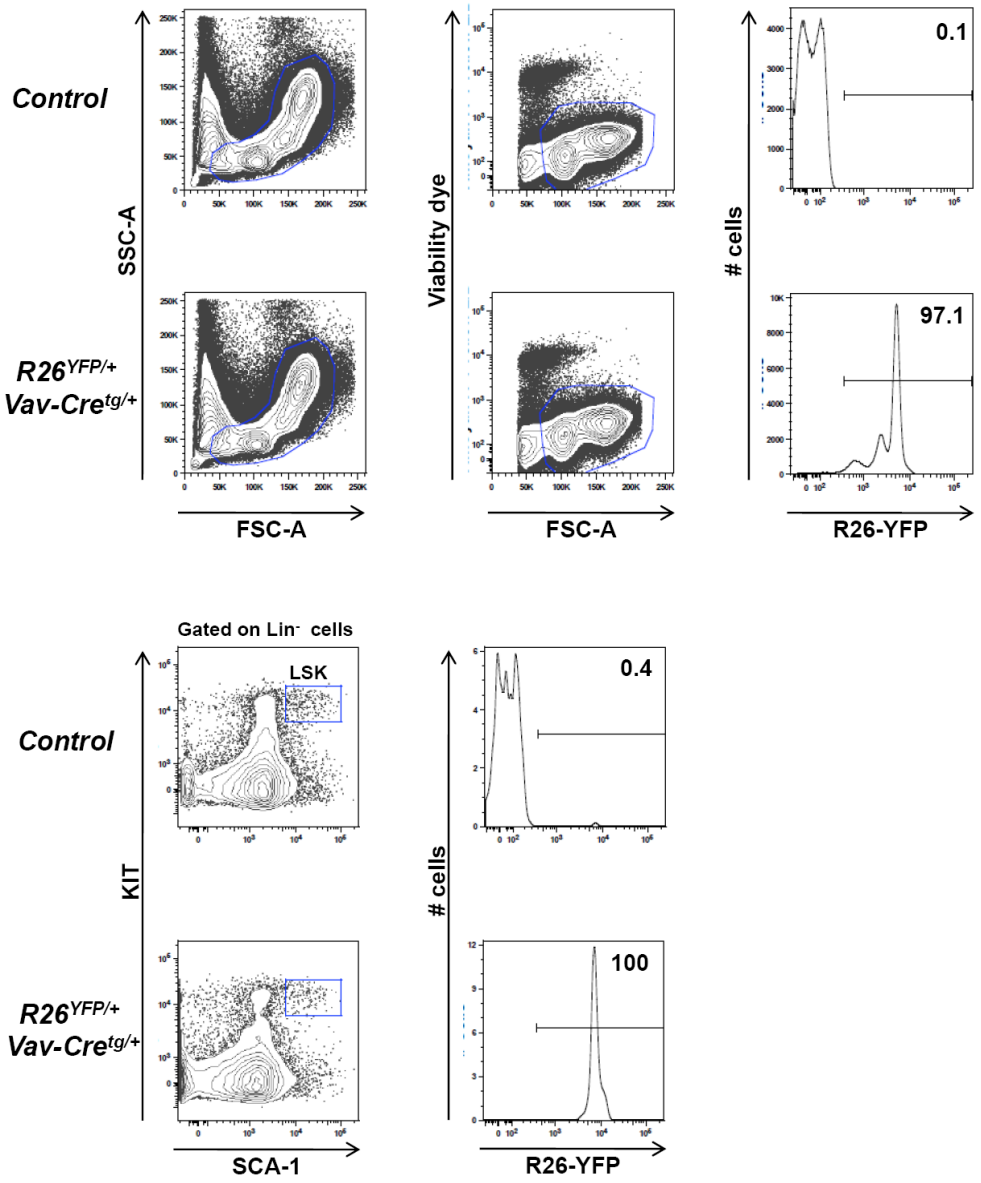
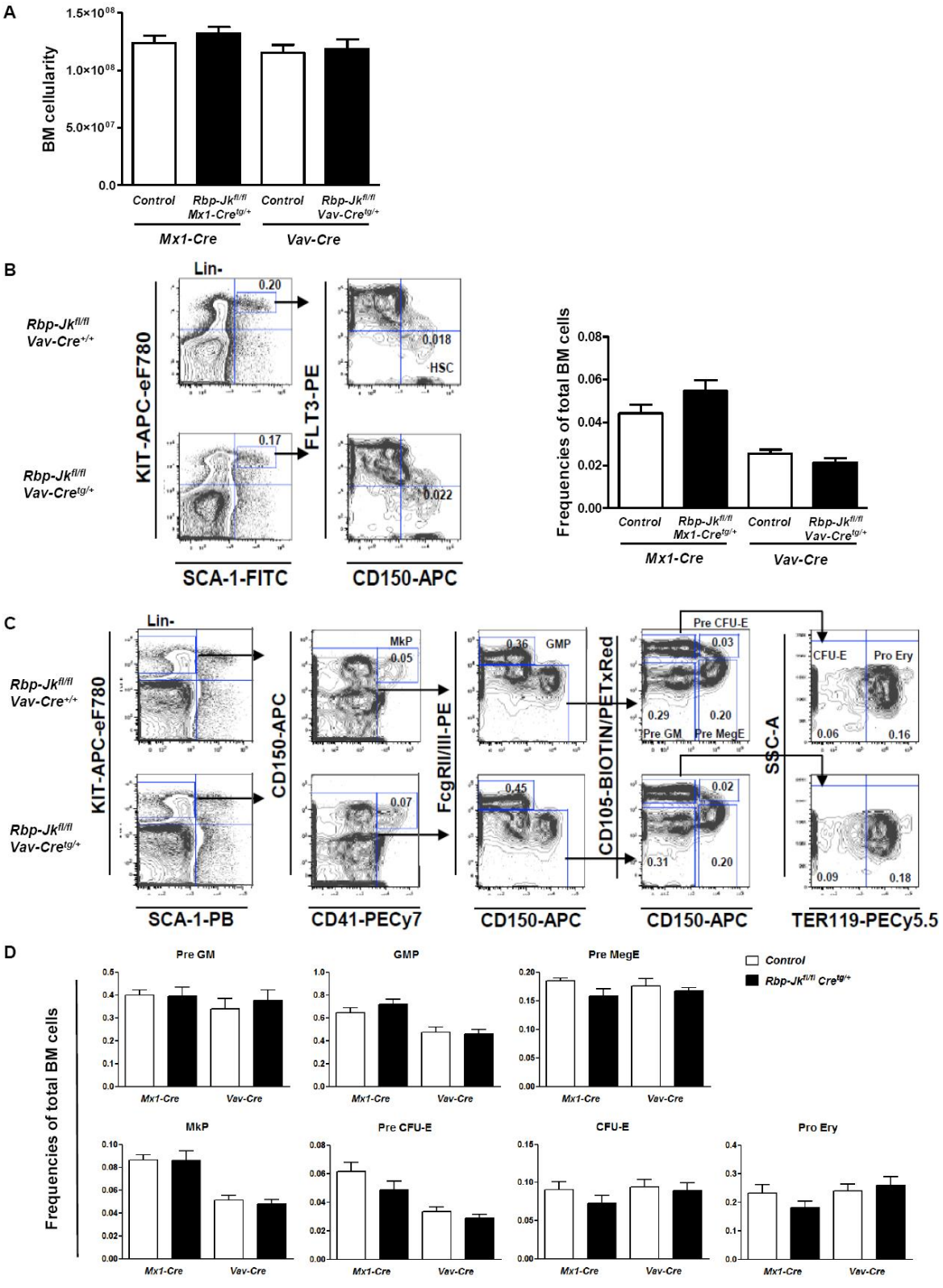


Figure 2.1. Vav-Cre is a strong Cre-recombinase inducer in the haematopoietic system.

Fourteen weeks-old *Rosa26*$^{YFP/+}$ *Vav-Cre*$^{tg/+}$ (N=1) and 10 weeks old *Rosa26*$^{YFP/+}$ *Vav-Cre*$^{+/+}$ control (N=1) mice were analyzed for YFP expression in unfractionated BM (upper panels) as well as Lin^{-}SCA-1^{+}c-KIT^{+} (LSK) population (lower panels) by FACS. Representative FACS profiles with percentages are shown.

BM cellularities were next measured for both *Rbp-Jk Mx1-Cre* and *Rbp-Jk Vav-Cre* lines, and no significant differences between *Rbp-Jk*-deficient and control mice were observed (Figure 2.2A). I then sought to evaluate the frequencies of Lin^- $Sca\text{-}1^+c\text{-}Kit^+CD150^+Flt3^-$ HSCs (Figure 2.2B) and the different subsets of myeloid progenitors as defined by Pronk and colleagues (Pronk et al., 2007) in *Rbp-Jk*-deficient and control mice (Figure 2.2C and 2.2D). In contrast with some studies (Duncan et al., 2005; Varnum-Finney et al., 2011) but in agreement with others (Maillard et al., 2008), BM HSC frequencies were not affected by the loss of *Rbp-Jk* (Figure 2.2B). Furthermore, applying a detailed FACS staging of GM, Mk and E progenitors (Pronk et al., 2007), I demonstrated that the frequencies of all myeloid progenitors were not affected in *Rbp-Jk*-deficient BM compared to controls. Importantly and in keeping with the recent study implicating Notch in Mk differentiation, frequencies of progenitors in the Mk hierarchy – PreMegEs and MkPs – did not alter in the absence of Notch signaling in $Rbp\text{-}Jk^{fl/fl}$ mice crossed to either $Mx1\text{-}Cre^{tg/+}$ or $Vav\text{-}Cre^{tg/+}$ lines (Figure 2.2C and 2.2D). To rule out a lack of phenotype due to the presence of BM progenitors that escaped Cre-mediated *Rbp-Jk* deletion, HSCs and myeloid progenitors were FACS sorted at high levels of purity and the deletion efficiency of the *Rbp-Jk* gene was assessed. As shown in figure 2.2E, recombination efficiency in all cell subsets in *Rbp-Jk*-deficient mice was highly efficient (>99% in all populations), independently of the Cre system used (Figure 2.2E). In agreement with this data, GM, Mk and E *in vitro* differentiation potential of BM cells harvested from mice lacking *Rbp-Jk* was also

unaffected as shown by comparable numbers of colonies formed in *Rbp-Jk*-deficient and control mice (Figure 2.2F and 2.2G). In addition, counts of blood circulating platelets were not affected upon loss of *Rbp-Jk.* Overall, the data presented here strongly suggest that Notch signaling is not required for the steady state maintenance or differentiation of HSCs nor myeloid progenitors in the BM.


A
BM cellularity
1.5×10^08
1.0×10^08
5.0×10^07
0.0
Control
Rbp-Jk^fl/fl Mx1-Cre^tg/+
Control
Rbp-Jk^fl/fl Vav-Cre^tg/+
Mx1-Cre
Vav-Cre
B
Lin-
Rbp-Jk^fl/fl Vav-Cre^+/+
Rbp-Jk^fl/fl Vav-Cre^tg/+
KIT-APC-eF780
FLT3-PE
0.20
0.018
HSC
0.17
0.022
SCA-1-FITC
CD150-APC
Frequencies of total BM cells
0.08
0.06
0.04
0.02
0.00
Control
Rbp-Jk^fl/fl Mx1-Cre^tg/+
Control
Rbp-Jk^fl/fl Vav-Cre^tg/+
Mx1-Cre
Vav-Cre
C
Lin-
Rbp-Jk^fl/fl Vav-Cre^+/+
Rbp-Jk^fl/fl Vav-Cre^tg/+
KIT-APC-eF780
SCA-1-PB
CD150-APC
MkP
0.05
0.07
CD41-PECy7
FcgRII/III-PE
0.36
GMP
0.45
CD150-APC
CD105-BIOTIN/PETxRed
Pre CFU-E
0.03
0.29
Pre GM
0.20
Pre MegE
0.02
0.31
0.20
CD150-APC
SSC-A
CFU-E
Pro Ery
0.06
0.16
0.09
0.18
TER119-PECy5.5
D
Frequencies of total BM cells
Pre GM
GMP
Pre MegE
Control
Rbp-Jk^fl/fl Cre^tg/+
MkP
Pre CFU-E
CFU-E
Pro Ery
Mx1-Cre
Vav-Cre

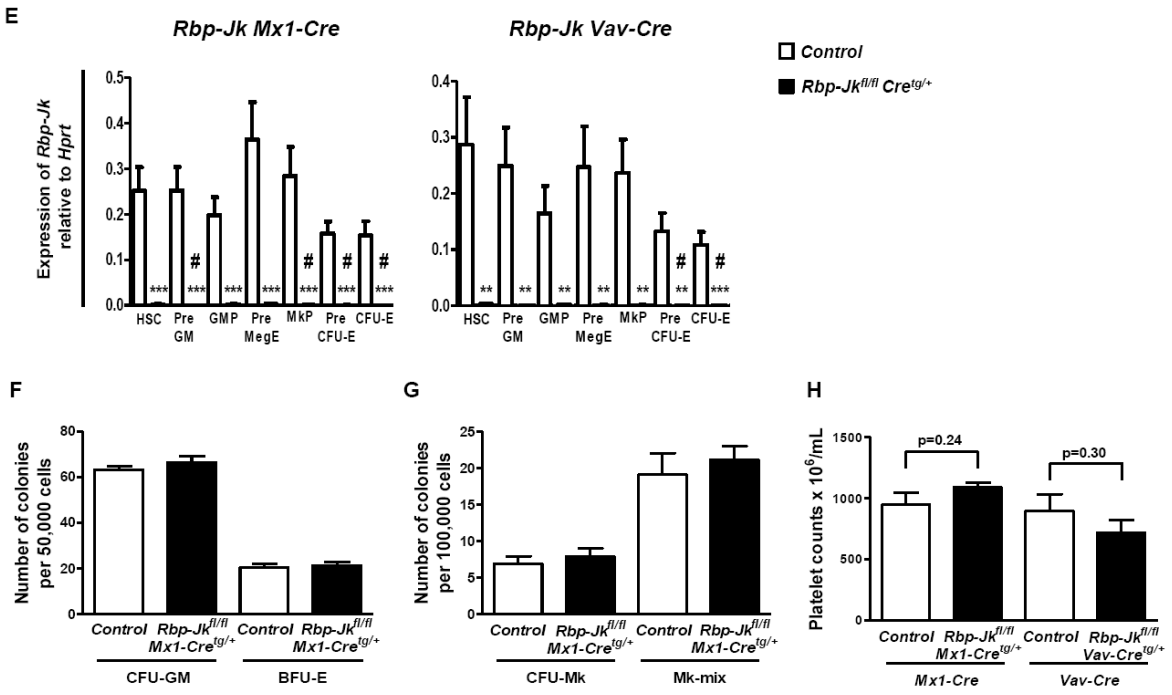


Figure 2.2. Myeloerythroid progenitors are unaffected in steady state BM of *Rbp-Jk*-deficient mice

(A-D) Adult poly(I:C)-treated *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ and age-matched controls (*Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Mx1-Cre*$^{tg/+}$) and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice and age-matched controls (*Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Vav-Cre*$^{tg/+}$) were analyzed. **(A)** Mean (SEM) BM cellularity per two femurs and two tibias (N=6-12). (**B)** Representative FACS profiles and mean (SEM) frequencies of LSKCD150^{+}FLT3^{-} HSCs and **(C and D)** myeloid progenitors in *Rbp-Jk Mx1-Cre* (N=13-14) or *Rbp-Jk Vav-Cre* (N=6-8) genotypes. **(E)** HSCs and myeloid progenitor subsets were sorted from individual *Rbp-Jk Mx1-Cre* (N=4-6) or *Rbp-Jk Vav-Cre* (N=3-5) mice and expression of *Rbp-Jk* analysed. Mean gene expression values normalized to *Hprt*. For all experiments, 3-6 mice per genotype and cell population (100 cells per replicate) were investigated individually. Samples in which the mean value of replicates was ≤0.001 (relative to *Hprt* expression) were considered below cut-off value (#). **(F)** *In vitro* colony forming unit granulocyte-monocyte (CFU-GM) and erythroid (BFU-E) colony formation of BM from *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ (N=4-5) and age-matched controls

(N=6-8). **(G)** Pure megakaryocytic (CFU-Mk) and Mk mixed lineage (Mk-mix) colonies were scored after staining with Acetylthiocholiniodide. Mean (SEM) values from 2-3 experiments (2 replicates per mouse). **(H)** Mean (SEM) circulating platelet counts in *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ (N=6) and matched controls (N=7) and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=10) and controls (N=13).

* p<0.05, ** (p <0.01), *** (p <0.001), otherwise no significant differences between *Rbp-Jk*-deficient and control groups.

2.2.2 *Rbp-Jk* deficiency does not affect key transcriptional myeloerythroid lineage programs in HSCs or myeloid progenitor subsets.

I next investigated whether the typical pattern of myeloerythroid gene expression characteristic of HSCs (Hu et al., 1997; Mansson et al., 2007) and myeloerythroid progenitors as previously shown (Mercher et al., 2008), are affected by the absence of canonical Notch signaling. To pursue this, levels of expression of GM and Mk/E related genes in HSCs and myeloid progenitors from both *Rbp-Jk*-deficient and control mice were measured. The transcript levels of the key GM-related genes, *granulocyte-macrophage colony-stimulating factor receptor alpha* (*Gm-csf receptor alpha)* and *granulocyte colony-stimulating factor receptor (G-csf receptor), Cebpα* and *Myeloperoxidase (Mpo)* were comparable between *Rbp-Jk*-deficient and control groups. Similarly, the expression of the Mk/E specific genes *stem cell leukaemia haematopoietic transcription factor (Scl), Gata1*, *zinc finger protein 1* (*Fog1)*, *ETS variant gene 6 (Etv6/Tel-1)*, *Gata2*, *nuclear factor erythroid 2*

(*Nfe2*), *Kruppel-like factor 1 (Klf1*), *erythropoietin receptor (Epor), Mpl* and *von Willebrand factor* (*Vwf*) in the *Rbp-Jk*-deficient BM cell subsets assessed were similar to controls (Figure 2.3). These results further support the view that canonical Notch signaling has no role in any stage of myelopoiesis.

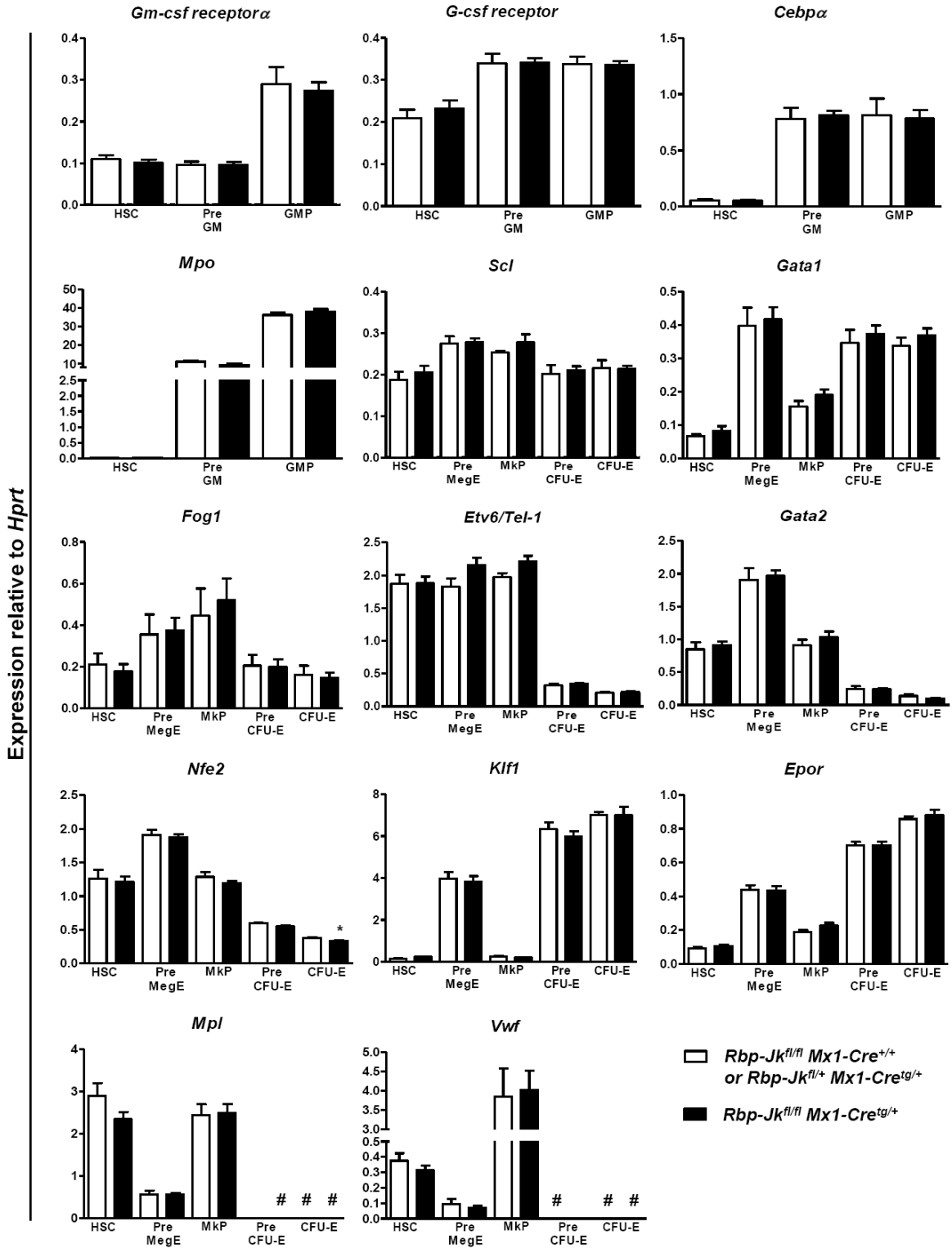


Figure 2.3. Transcriptional expression of myeloerythroid lineage programs are not affected in HSCs or myeloid progenitors at distinct stages in *Rbp-Jk*-deficient mice

LSKCD150^{+}Flt3^{-} HSCs and myeloid progenitor subsets purified from individual adult *Rbp-Jk$^{fl/fl}$ Mx1-Cre$^{tg/+}$* (N=6) and age-matched *Rbp-Jk$^{fl/fl}$ Mx1-Cre$^{+/+}$* or *Rbp-Jk$^{fl/+}$ Mx1-Cre$^{tg/+}$* control (N=4) poly(I:C) treated mice, were subjected to quantitative gene expression analysis for myeloid (*Gm-csf receptor α, G-csf receptor*, *Cebpα*, and *Mpo*) and Mk/E (*Scl, Gata1*, *Fog1*, *Etv6/Tel-1*, *Gata2*, *Nfe2*, *Klf1*, *Epor*, *Mpl* and *Vwf*) related genes. Mean gene expression values normalized to *Hprt*. For all results, 4-6 mice per genotype and cell population (100 cells per replicate) were investigated individually. Samples in which the mean value of replicates was ≤0.001 (relative to *Hprt* expression) were considered below cut-off value (#).* $p<0.05$

2.2.3 RBP-Jk is not required for expansion or maintenance of BM stem cell or myeloid progenitor compartments after transplantation

We next posed the question as to whether Notch signaling could be important for myeloid development under conditions of great stress, such as after myeloablation followed by BM transplantation. Recipient mice were lethally irradiated and reconstituted with one million of *Rbp-Jk$^{fl/fl}$ Mx1Cre$^{tg/+}$* or *Rbp-Jk$^{fl/fl}$Cre$^{+/+}$* control unfractionated BM cells along with the same amount of wild type competitor BM cells. As shown in figure 2.4, *Rbp-Jk*-deficient cells showed no advantage or disadvantage in reconstituting the HSC (Figure 2.4A) and myeloid (Figure 2.4B) compartments of the recipients.

In the Mercher *et al* study, platelets were not affected in mice non-competitively transplanted with *dnMAML*-transduced LSK cells, although MEPs were reduced (Mercher et al., 2008). However, an effect on platelet numbers might

have been missed, since a competitive assay setting was not performed (Mercher et al., 2008). In my experiments, the reconstitution of donor platelets in competitively transplanted mice was not affected in mice transplanted with *Rbp-Jk*-deficient cells compared with controls (mean donor platelet counts of 21.3% and 16.8% in the wild type and *Rbp-Jk*-deficient groups, respectively, p value 0.40; Figure 2.4C). Thus, these data support the view that canonical Notch signaling is also dispensable for maintenance and expansion of HSCs and myeloerythroid progenitors in conditions of distress where cell turnover occurs much more rapidly, such as after transplantation.

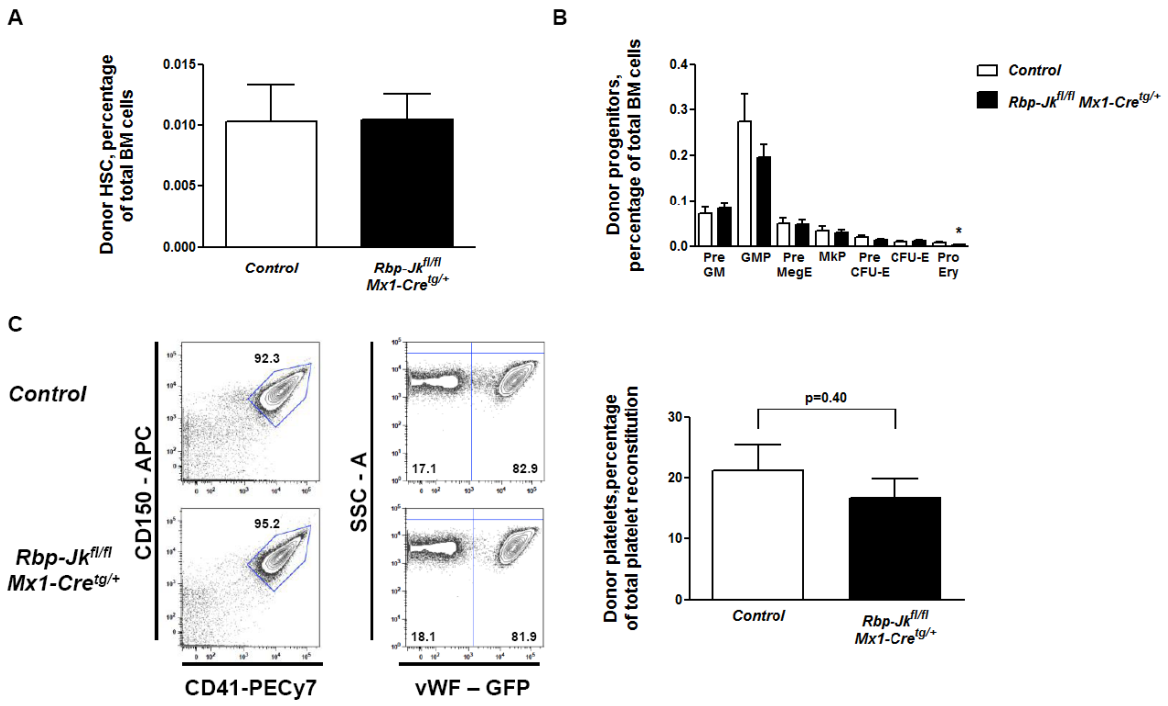


Figure 2.4. Canonical Notch signaling is dispensable for reconstitution of HSCs and myeloerythroid progenitors after transplantation

Lethally irradiated CD45.1 or CD45.1/2 recipient mice (N=5-12) were competitively transplanted (1:1) with BM cells from poly(I:C)-treated CD45.2 *Rbp-Jk Mx1-Cre* transgenic mice. Reconstitution of **(A)** LSKCD150^{+}Flt3^{-} HSCs and **(B)** myeloid progenitor subsets in BM of engrafted mice 7-9 weeks after transplantation. Mean (SEM) values.

(C) Reconstitution of donor blood platelets (CD41^{+}CD150^{+}GFP^{-}) in *vWF2-eGFP* CD45.1/2 recipients 5 weeks after transplantation with 1 million *Rbp-Jk$^{fl/fl}$ Mx1-Cre$^{tg/+}$* (N=8) or control *Rbp-Jk$^{fl/fl}$ Mx1-Cre$^{+/+}$* or *Rbp-Jk$^{fl/+}$ Mx1-Cre$^{tg/+}$* (N=8) BM cells and equal numbers of *vWF2-eGFP* CD45.1/2 competitor BM cells. Shown to the left are representative FACS profiles of platelet reconstitution from engrafted mice. To the right, mean (SEM) donor platelet reconstitution of all mice.

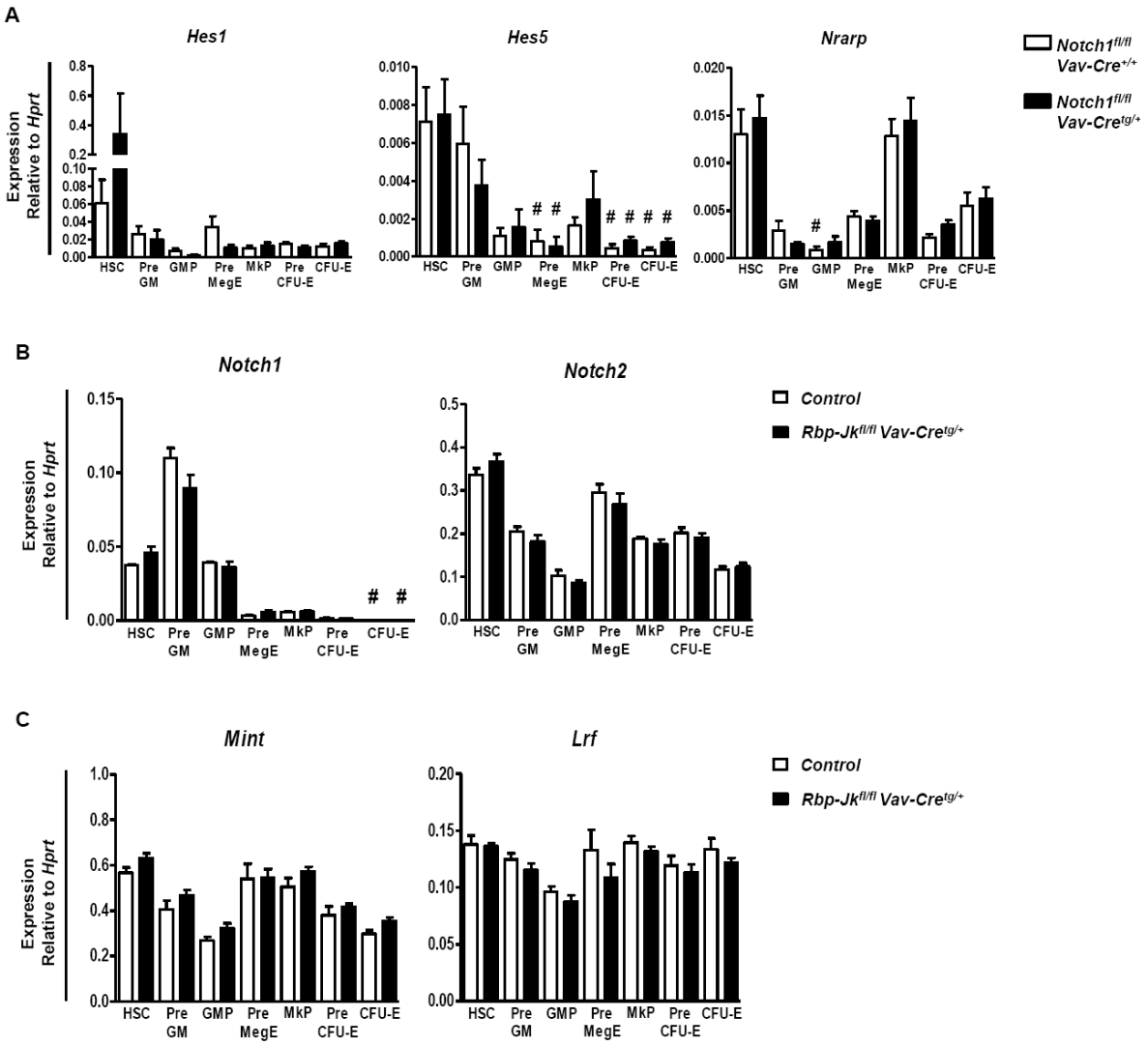
* $p<0.05$, otherwise no significant differences between *Rbp-Jk*-deficient and controls.

2.2.4 Notch target genes are kept in a suppressed state by RBP-Jk in BM myeloid progenitors.

To further resolve whether Notch plays a role, if any, in the regulation of HSCs and myeloerythroid progenitors, I next sought to investigate whether activation of relevant Notch target genes is dependent upon Notch signalling at these stages of myeloerythropoiesis in the BM.

Mice genetically modified to carry a *Notch1* gene with *LoxP* sites flanking a 3.5 Kb gene segment containing part of the putative *Notch1* promoter and the exon

encoding the signal peptide preceding the EGF repeats in *Notch1* gene were used (Radtke et al., 1999). *Notch1*$^{fl/fl}$ mice were bred to *Vav-Cre* transgenics and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ mice were studied. First, the levels of messenger ribonucleic acid (mRNA) transcripts of the key Notch target genes, *Hes1, Hes5* and *Nrarp,* in *Notch1*-deficient HSCs and stages of myeloid progenitors were assessed, and no effect on gene expression levels was observed in *Notch1*-deficient mice compared to controls (Figure 2.5A). In contrast, in the absence of *Rbp-Jk*, whereas gene expression of *Notch1* and *Notch2* (figure 2.5B) as well as Notch signaling inhibitors, *Mint* and *Lrf* (Figure 2.5C), were not affected compared to controls, the direct Notch target genes, *Hes1, Hes5* and *Nrarp,* were upregulated upon loss of *Rbp-Jk,* using either Mx1-Cre or Vav-Cre approaches (Figure 2.5D). The levels of expression of the same Notch target genes were measured in thymic DN2 and DN3 cells from *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Mx1-Cre*$^{tg/+}$ as positive controls for the assay and were, as expected, readily expressed (Figure 2.5D). Overall, these data demonstrate for the first time in primary mammalian cells *in vivo* that RBP-Jk suppresses Notch target genes when Notch signaling is off.


A
Hes1
Hes5
Nrarp
Expression Relative to Hprt
Notch1fl/fl Vav-Cre+/+
Notch1fl/fl Vav-Cretg/+
HSC
Pre GM
GMP
Pre MegE
MkP
Pre CFU-E
CFU-E
B
Notch1
Notch2
Control
Rbp-Jkfl/fl Vav-Cretg/+
C
Mint
Lrf

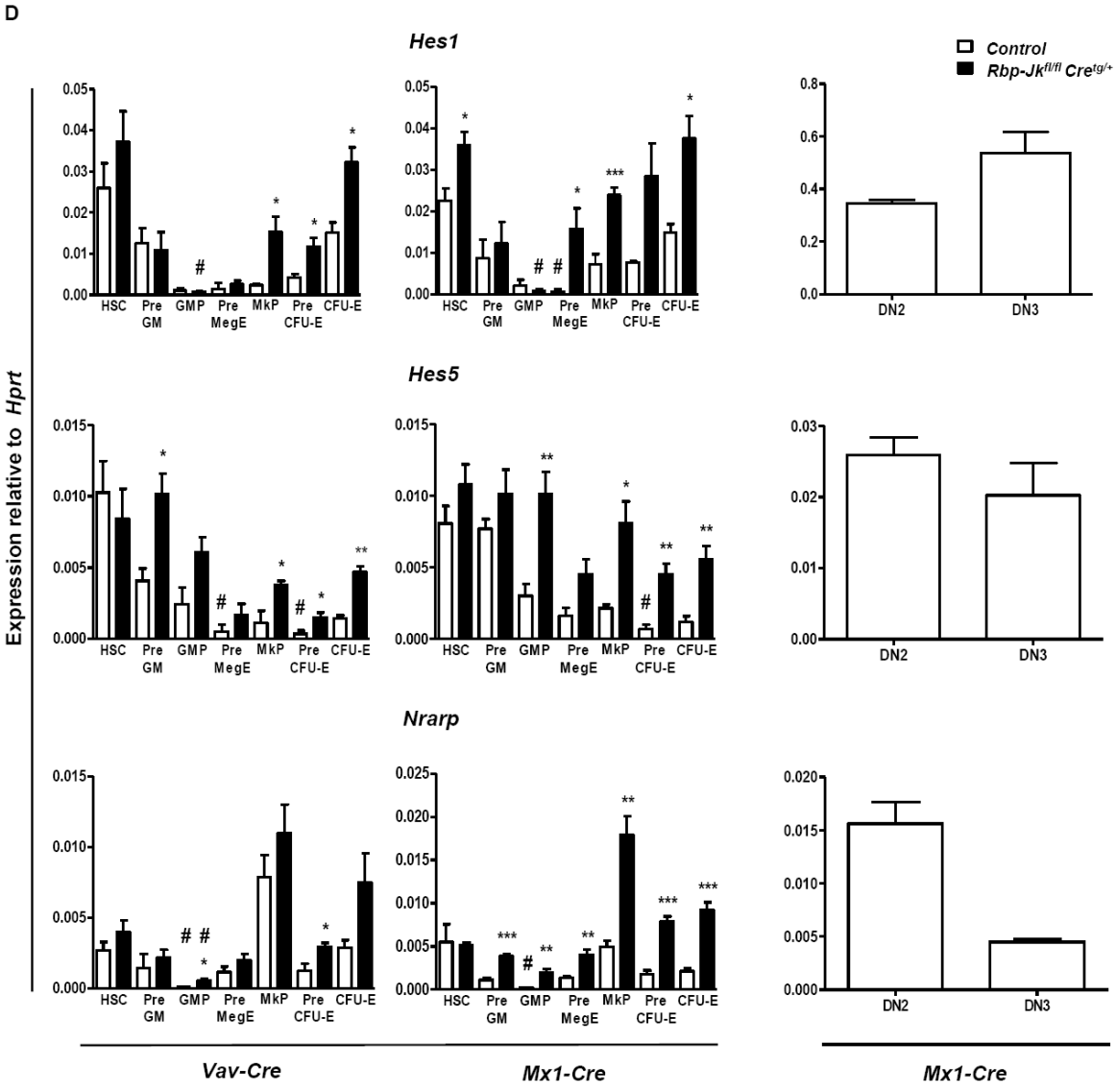


Figure 2.5. Notch target genes are kept in a suppressed state by RBP-Jk in megakaryocyte and erythroid progenitor cells

(A) BM HSCs and myeloid progenitor subsets were purified from individual adult *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=4) and control *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ (N=4) mice and analysed quantitatively for gene expression analysis of *Hes1*, *Hes5* and *Nrarp*.

Rbp-Jk$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and age-matched *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ controls were analysed for expression of **(B)** *Notch1* and *Notch2*, **(C)** Notch inhibitors *Mint* and *Lrf* and **(D)** Notch target genes *Hes1*, *Hes5* and *Nrarp***,** in BM stem and myeloid progenitor

cells. The same analysis of Notch target genes was done on BM HSCs and myeloid progenitors sorted from poly(I:C) treated *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ and age-matched *Rbp-Jk*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ or *Rbp-Jk*$^{fl/+}$ *Mx1-Cre*$^{tg/+}$ controls (middle panels). For comparison, expression levels were investigated in c-KIT^{+}DN2 and DN3 thymic progenitors (right panels). For all panels, 3-5 mice per cell population and genotype (100 cells per replicate) were investigated individually. Mean (SEM) values are shown. Samples in which the mean value of replicates was ≤0.001 (relative to *Hprt* expression) were considered below cut-off value (#). * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

2.3 Discussion

The role of Notch is very well established to be essential for the generation of definitive HSCs during embryonic development (Kumano et al., 2003) as well as for T cell development in the thymus, but its role in postnatal haematopoiesis in the BM remains disputed.

Herein, it is unequivocally demonstrated that canonical Notch signaling is not necessary for myeloid cell fate determination and lineage restriction events in the BM. Through a detailed staging of the myeloid populations in the BM by FACS (Pronk et al., 2007), my studies show that Notch signaling is dispensable for HSC maintenance and expansion and also that the numbers of distinct myelo-erythroid progenitors, including multiple stages of Mk progenitors, are not affected by the absence of *Rbp-Jk*. Furthermore, the function and growth of BM progenitors is unaltered in the absence of Notch signaling in steady state as well as upon

conditions of stress, such as after transplantation, further demonstrating no role for Notch signaling in the proliferation and differentiation of myeloid progenitors of all lineages in the BM. It was also observed that the expression of key genes comprising the myeloid and MkE lineage signatures are not affected upon deletion of *Rbp-Jk*, further supporting Notch signaling unnecessary for myeloerythroid cell fate specification in the BM. My conclusions are in contrast with those of Mercher and colleagues, who proposed Notch signaling to specify megakaryopoiesis from HSCs (Mercher et al., 2008), perhaps at the expense of the other myeloid lineages. In their study, they reported that MEPs were dramatically reduced in *dnMAML1-EGFP* transgenic mice. In addition, gene expression analysis revealed that wild type MEPs express the Notch target genes, *Hes1* and *Hey1,* at higher levels than the other myeloid progenitors (CMP and GMP), suggesting Notch signaling activation in the Mk and E lineages (Mercher et al., 2008). Although the cause of the discrepancy between the studies herein reported and Mercher *et al* studies is not clear, one should keep in mind that in the Mercher *et al* study a transgene is introduced into the cell to abrogate Notch signalling, which could have effects on cellular processes unrelated to Notch signalling that may affect the phenotype observed. For instance, MAML1 has been shown to act on p53, β-catenin and MEF2C pathways (McElhinny et al., 2008) and therefore it is possible that DNMAML1, in contrast to the "clean" *Rbp-Jk* deletion used here, may affect signaling pathways other than Notch signaling. Alternatively, the effect of DNMAML1 in Mk development could result from non-canonical Notch signaling. However this hypothesis is unlikely as it is well established that DNMAML1

specifically inhibits Notch signaling through the canonical RBP-Jk-NICD-MAML complex (Maillard et al., 2004). Furthermore, although observed in *Drosophila,* where the phenotype of *Notch1* (the single Notch receptor identified in flies) and *Su(H)* mutants were not identical, but rather stronger in the Notch mutants (Rusconi and Corbin, 1998, 1999; Zecchini et al., 1999), non-canonical Notch signaling has never been convincingly demonstrated in mammals.

In the present studies it is further demonstrated, for the first time in primary mammalian cells *in vivo,* that Notch target genes are kept in a suppressed state by RBP-Jk in the BM. My data show that the transcription levels of the key Notch target genes, *Hes1, Hes5* and *Nrarp,* in myeloid progenitors, particularly of the Mk and E lineages, are upregulated in the absence of *Rbp-Jk* and that this upregulation is NOTCH1-independent, since the transcription levels of the same target genes are unaltered in *Notch1*-deficient BM progenitors. In fact, the repressive function of CSL was first demonstrated in a study in flies by Bailey and co-workers (Bailey and Posakony, 1995), which reported that the transcriptional activation of Notch target genes in the E(spl) complex increases in the absence of *Su(H).* A more recent study in mouse embryonic fibroblasts, demonstrated that the Notch target gene, *Hey1,* is derepressed in the absence of *CtBP*, a corepressor recruited by the Rbp-Jκ/SHARP complex to silence Notch target genes (Oswald et al., 2005), supporting the hypothesis that Rbp-Jk functions as a transcriptional repressor of Notch target genes. The role of this repressed state is not obvious, in particular in my studies as there is no obvious impact of deleting *Rbp-Jk* on the numbers and function of myeloid progenitor cells. However it should be

considered that suppression of Notch target genes in BM progenitors may prevent ectopic T cell development in the BM, as this normally only occurs in the thymus. Furthermore, in contrast with a recent study in *Drosophila*, where Su(H) is recruited to DNA only when NICD is present (Krejci and Bray, 2007), data shown here support the view that RBP-Jk is positioned on DNA in the absence of Notch signaling, and therefore, the NICD.

Overall, studies herein demonstrate that canonical Notch signaling is not important for myeloid progenitor maintenance or lineage specification in the BM, and that RBP-Jk functions as an active transcriptional repressor of Notch target genes in BM progenitors, possibly restricting T cell development to the thymus.

2.4 Future directions

2.4.1 Molecular mechanism underlying Notch target gene upregulation in *Rbp-Jk*-deficient mice

In view of the observation that key Notch target genes are upregulated in BM progenitors in the absence of *Rbp-Jk*, it will be important to understand how this gene expression fluctuation happens at the molecular level. Where, on the gene promoter, does RBP-Jk exert its suppressive role? Is it RBP-Jk that has a direct suppressive function or is it one of the co-repressors in the repressive complex? Thus, an extensive molecular study, incluiding DNA-protein interaction assays, such as Chromatin immunoprecipitation (Chip)-on-Chip or Chip sequencing and genome wide expression profiling, should be the next step trying

to understand how Rbp-Jk-mediated gene expression regulation happens in the BM in mammals. Likewise, it will be important to understand the functional impact of the RBP-Jk-mediated gene suppression on BM progenitor cells.

2.4.2 Is Notch target gene activation observed in Rbp-Jk-deficient mice consequential to non-canonical Notch signaling?

The existence of non-canonical Notch signaling in mammals remains to be established, and therefore finding an answer to the question above is, currently, technically very difficult. Nevertheless, it would be of key importance to understand whether the observed upregulation of Notch target genes results from a RBP-Jk-independent signaling pathway. One way of evaluating the existence of non-canonical Notch signaling would be to have a *Notch1*$^{-/-}$*Nocth2*$^{-/-}$*Notch3*$^{-/-}$*Notch4*$^{-/-}$ quadruple knockout mouse model. However mouse genetic engineering has not evolved to the stage of generating such a mouse yet. An alternative would be to compare global gene expression in wild type and *Rbp-Jk* deleted cells previously exposed to Notch ligands, to identify candidate genes activated through non-canonical Notch signaling.

3

The role of Notch in regulation of early thymic progenitors

3.1 Introduction

Mx1-Cre mediated deletion of *Notch1* and *Rbp-Jk* in mice leads to an expansion of $Lin^{-}CD44^{+}CD25^{-}$ DN1 cells and a block between the DN1 to DN2 stages (Han et al., 2002; Radtke et al., 1999). Phenotypic analysis of expanded DN1 cells revealed that these cells have a (BM) B cell-like phenotype (Han et al., 2002; Radtke et al., 1999). Moreover, ETPs are found within the heterogeneous DN1 thymic population and are characterized by expression of high levels of the c-KIT receptor (Allman et al., 2003). These progenitors are hardly found in the *Notch1-Mx1Cre* mice (Benz et al., 2008), and their generation from TSPs is dependent upon Notch signaling (Sambandam et al., 2005; Tan et al., 2005). In contrast *Notch2*- and *Notch3*-deficient mice do not have an ETP phenotype (Krebs et al., 2003; Saito et al., 2003), suggesting that NOTCH1 is the main Notch receptor implicated in early T cell development in the thymus, which can compensate for the absence of the other Notch receptors and is essential for the establishment of ETPs.

Furthermore, expression levels of the Notch target genes, *Dtx1*, *Hes1*, *Gata3* and *Cd25,* increase from HSCs to ETPs (Sambandam et al., 2005; Tan et al., 2005). However, it remains unclear whether this gene activation is indeed Notch-dependent and whether Notch signaling is required for the migration of TSPs to the thymus or only for the generation of ETPs once the progenitors have seeded that organ. In addition, the identity of the first cell receiving Notch signals as well as where these signals are first delivered, BM or thymus, remains unknown.

3.2 Results

3.2.1 NOTCH1 receptor is dispensable for the establishment of ETPs but essential for the DN1-DN2 transition in the thymus of *Notch1$^{fl/fl}$ Vav-Cre$^{tg/+}$* newborn mice

First I aimed to investigate the role of NOTCH1 in the establishment of ETPs in the thymus. T cell development in the thymus is particularly active very early in life (Hollander et al., 2006) and data from our laboratory shows that multipotent ETPs are detected at higher frequencies in neonates (Luc S *et al*, 2011, manuscript submitted). However, *Notch1*-deficient mice die during embryonic genesis due to abnormalities in somitogenesis (Conlon et al., 1995). *Vav-Cre*-mediated gene inactivation starts around day e10 in the embryo (Stadtfeld and Graf, 2005), immediately after HSCs have been generated, and therefore we hypothesized that *Notch1* deletion should not interfere with the critical role of NOTCH1 in the emergence of adult HSCs in the embryo (Chen et al., 2009; Kumano et al., 2003). Hence, in order to study the role of Notch in ETP establishment in the thymus, *Notch1$^{fl/fl}$* mice were bred to *Vav-Cre$^{tg/+}$* mice.

Notch1 gene inactivation in *Notch1$^{fl/fl}$ Mx1-Cre$^{tg/+}$* neonates leads to growth retardation that is reflected by lower body weight compared to control mice (Radtke et al., 1999). In contrast, the body weight of *Notch1$^{fl/fl}$ Vav-Cre*$^{tg/+}$ mice was comparable to *Notch1$^{fl/fl}$ Vav-Cre$^{+/+}$* control littermates at 4 and 8-10 days of age

(Figure 3.1A), suggesting that Vav-Cre-mediated deletion of *Notch1* is associated with fewer side effects.

Thymi were harvested from *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ littermates up to ten days of age. As shown in figure 3.1B, thymic cellularity was 6.5-fold reduced in the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice compared to controls, in agreement with reports of *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice (Radtke et al., 1999), suggesting that NOTCH1 is important for T cell development in the thymus. However, and in contrast with previous reports, the frequency of Lin^{-} CD4^{lo}CD8^{lo}CD25^{-}c-KIT^{hi} ETPs was 2.5-fold increased in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ compared to *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ control mice (Figure 3.1C), and consequently absolute numbers of ETPs in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice were only 2.6-fold reduced compared to controls (Figure 3.1D). Furthermore, FACS analysis showed that the number of thymic B220^{+} cells were expanded in the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice, supporting the generation of ectopic B cells in the thymus, as previously demonstrated in *Notch1*-deficient mice (Radtke et al., 1999) (Figure 3.1C). Importantly, c-KIT^{+} DN2 and DN3 progenitors were almost absent in *Notch1*-deficient mice compared to control littermates (Figure 3.1C and 3.1E), implicating a critical role of NOTCH1 in the DN1 to DN2 stage transition. Absolute numbers of mature CD4^{+}CD8^{+} double positive T cells were 12.7-fold reduced in *Notch1-Vav-Cre* mice (Figure 3.1F and 3.1G), most likely a consequence of the block in differentiation from DN1 to DN2 stage. Contrastingly, CD4^{-}CD8^{-} DN cells are overall increased in Figure 3.1G, which is due to the presence of increased number of B cells as well as other CD4/CD8 non-expressing cells (Figure 3.1G). Taken together these data demonstrate that NOTCH1 is not important for the generation

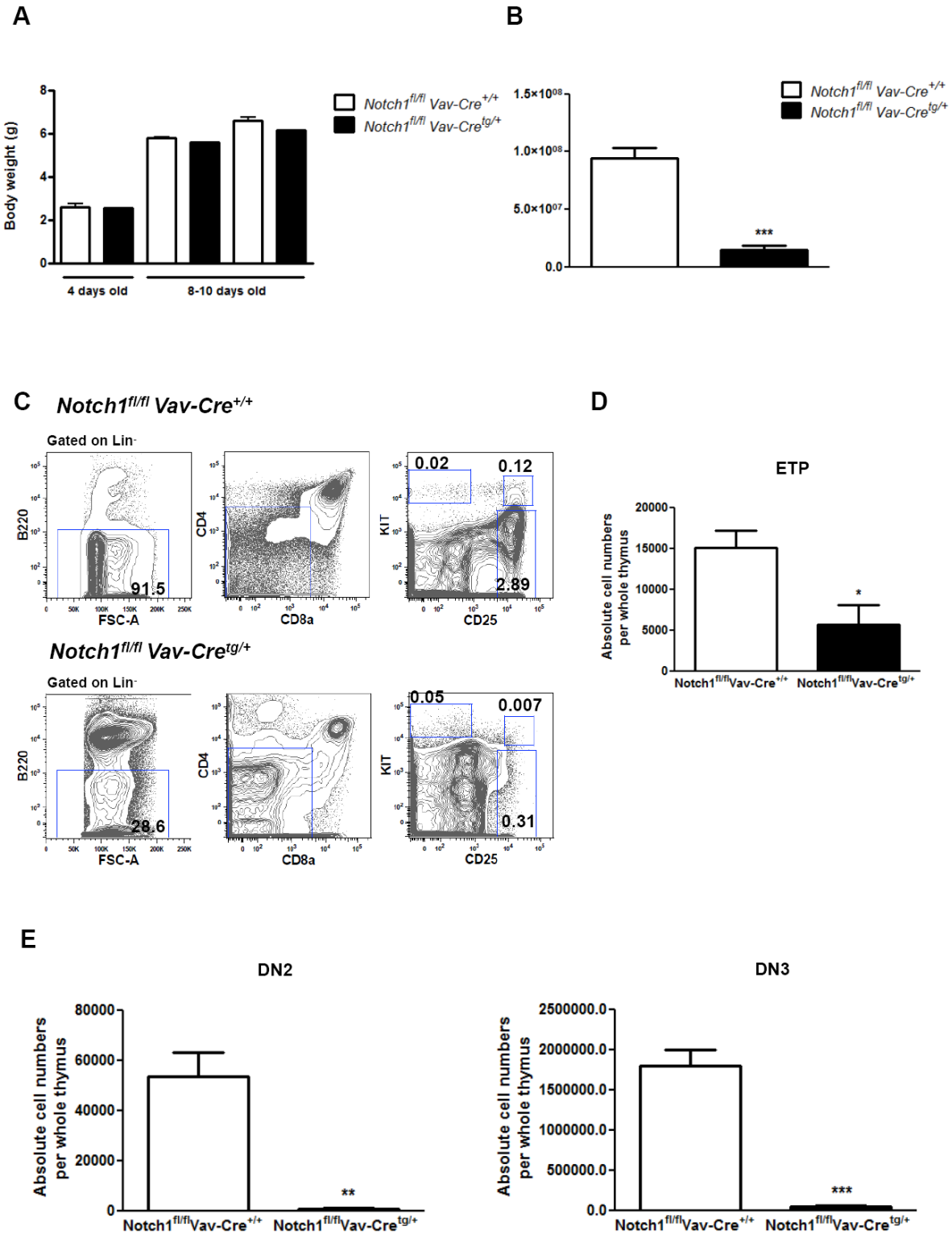
of ETPs but absolutely crucial for the differentiation into DN2 cells and downstream progenitors.

It was then sought to investigate whether the block in the DN1-DN2 transition could be explained by altered gene expression in ETPs. Thus, expression levels of the Notch target genes, *Cd25, Gata3, Nrarp, Hes1, Hes5, Runt-related transcription factor (Runx) 1 and Runx3,* were assessed in *Notch1*-deficient ETPs. Consistently, all genes were downregulated in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice compared to controls (Figure 3.1H), demonstrating Notch signaling activation as early as at the DN1 stage, supporting the view that the DN1-DN2 transition is *Notch1*-dependent.

So far, results presented here differ from previous reports where an essential role for NOTCH1 has been implicated already for ETPs in the thymus (Sambandam et al., 2005; Tan et al., 2005). To exclude the possibility that the less severe phenotype in the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ is due to cells that have escaped *Notch1* gene inactivation, *Notch1* gene expression was evaluated by the Quantitative-polymerase chain reaction (Q-PCR) in different thymic progenitors. *Notch1* was virtually absent in purified ETPs, and similarly, DN3, CD4 SP, CD8 SP and CD4^{+}CD8^{+} DP did not express any significant *Notch1* mRNA levels, confirming an efficient deletion of *Notch1* in ETPs and downstream progenitors (Figure 3.1I). Further, since the *Flt3* receptor is exclusively expressed by ETPs in the thymus and undetectable in the downstream T cell stages (Sambandam et al., 2005), the expression of *Flt3* was assessed in thymic T cell progenitors. As expected, *Flt3* mRNA was only detected in ETPs, in both *Notch1*-deficient and control groups, but

not in the downstream populations (Figure 3.2J). This further corroborates that the emergence of *bona fide Flt3*-expressing ETPs is largely *Notch1*-independent.

Collectively, the results described above demonstrate that, in contrast with previous reports, NOTCH1 is dispensable for the establishment of ETPs in the thymus but is critical for the transition from ETPs to DN2 cells.


A
Body weight (g)
4 days old
8-10 days old
Notch1fl/fl Vav-Cre+/+
Notch1fl/fl Vav-Cretg/+
B
***
C
Notch1fl/fl Vav-Cre+/+
Gated on Lin-
B220
FSC-A
91.5
CD4
CD8a
KIT
CD25
0.02
0.12
2.89
Notch1fl/fl Vav-Cretg/+
28.6
0.05
0.007
0.31
D
ETP
Absolute cell numbers per whole thymus
E
DN2
DN3
**
***

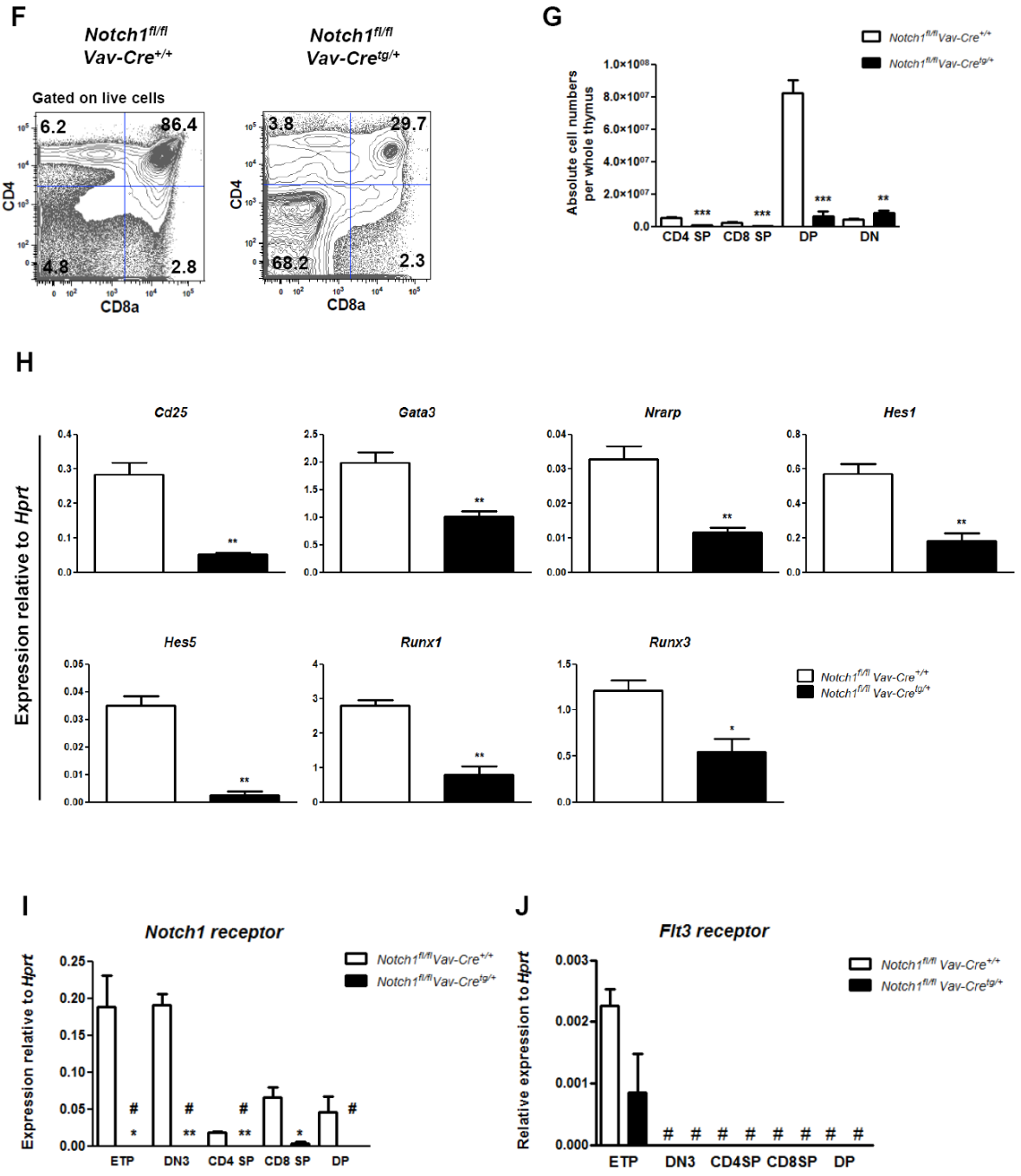


Figure 3.1. NOTCH1 is redundant for the establishment of ETPs in the thymus but crucial for downstream T cell development

(A) *Notch1 Vav-Cre* litters were genotyped and analysed at four-to-ten days of age. Mean (SEM) body weight of *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ control mice (N=1-2 and N=2-7 respectively).

(B) Four to ten days old *Notch1 Vav-Cre* mice were analysed. Mean (SEM) thymus cellularity (N=9 for the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and N=33 for the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Notch1*$^{fl/+}$ *Vav-Cre*$^{tg/+}$).

(C) Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^-$c-KIT^{hi} ETP frequencies were determined in the thymus of *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=9) and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Notch1*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ (N=33) control mice by FACS. Representative FACS profiles from *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ genotypes are shown. Numbers indicate mean percentages of total thymocytes within indicated gate.

(D) Mean (SEM) absolute cell number of ETPs in *Notch*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=9) and *Notch*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Notch*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ (N=33) control mice.

(E) Mean (SEM) absolute cell numbers of c-KIT$^+$ DN2 (Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^+$c-KIT$^+$) and DN3 (Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^-$c-kit$^-$) progenitor subsets in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=9) and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ or *Notch1*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ (N=33) control mice.

(F) Frequencies of mature CD4 SP, CD8 SP, CD4/CD8 DP and CD4/CD8 DN cells in *Notch1 Vav-Cre* mice were established by FACS. Representative FACS profiles from *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ genotypes. Numbers indicate mean percentage within indicated gate.

(G) Mean (SEM) absolute numbers of CD4 SP, CD8 SP, CD4/CD8 DP and CD4/CD8 DN cells in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ mice.

(H-J) ETPs were sorted from individual four-to-ten days old *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=3) and control *Notch1*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ (N=3) mice (100 cells per sample) and **(H)** gene expression levels using the Fluidigm Assay were determined for the Notch target genes *Cd25, Gata3, Nrarp, Hes1, Hes5, Runx1* and *Runx3.* **(I)** *Notch1 receptor* deletion efficiency and **(J)** expression of the ETP specific gene in the thymus *Flt3* was also determined by Fluidigm.

For all genes studied, mean gene expression values were normalized to *Hprt*. For all results, 4-6 mice per genotype and cell population (100 cells per replicate) were investigated individually. Samples in which the mean value of replicates was ≤0.001 (relative to *Hprt* expression) were considered to be below cut-off value (#).

* p<0.05, ** (p <0.01), *** (p <0.001), otherwise no significant differences between *Notch1*-deficient and control groups.

3.2.2 ETPs are absent in recombined *Notch1$^{fl/fl}$ Mx1-Cre$^{tg/+}$* neonates

In contrast to published results in *Notch1$^{fl/fl}$ Mx1-Cre$^{tg/+}$* mice, we have demonstrated that NOTCH1 is dispensable for the generation of ETPs in *Notch1$^{fl/fl}$ Vav-Cre$^{tg/+}$* mice. This discrepancy may be due to the negative side effects from the poly(I:C) treatment. In order to investigate that, *Notch1 Mx1-Cre* neonatal mice were studied. After establishing the optimal dose of poly(I:C) to achieve high Cre-mediated recombination in *Rosa26$^{YFP/+}$ Mx1-Cre$^{tg/+}$* and respective littermate neonates (data not shown), 3-6 days old *Notch1 Mx1-Cre* pups were injected intra-peritoneally with one single dose of 500ug of poly(I:C). Thymus cellularity was dramatically reduced 6-7 weeks after the poly(I:C) treatment in *Notch1*-deficient mice compared to controls (mean value of 44 million and 226 million cells per thymus in *Notch1$^{fl/fl}$ Mx1-Cre$^{tg/+}$* and *Notch1$^{fl/fl}$ Mx1-Cre$^{+/+}$* controls, respectively; Figure 3.2A). In agreement with previous reports (Benz et al., 2008; Radtke et al., 1999), the absolute numbers of ETPs, defined as Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^-$c-KIT^{hi}, were 12.9-fold reduced (Figure 3.2B and 3.2C). DN2 (Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^+$c-KIT^{hi}) were virtually absent and DN3 (Lin$^-$ B220$^-$CD4$^-$CD8$^-$CD25$^+$c-KIT$^-$) were

severely reduced (Figure 3.2B). Hence, in contrast with the phenotype observed in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$*,* but in agreement with published work, ETPs are very much reduced in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice, rather implicating negative effects on cell survival and differentiation of thymic cells from the poly(I:C) treated *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice (Kuhn et al., 1995).

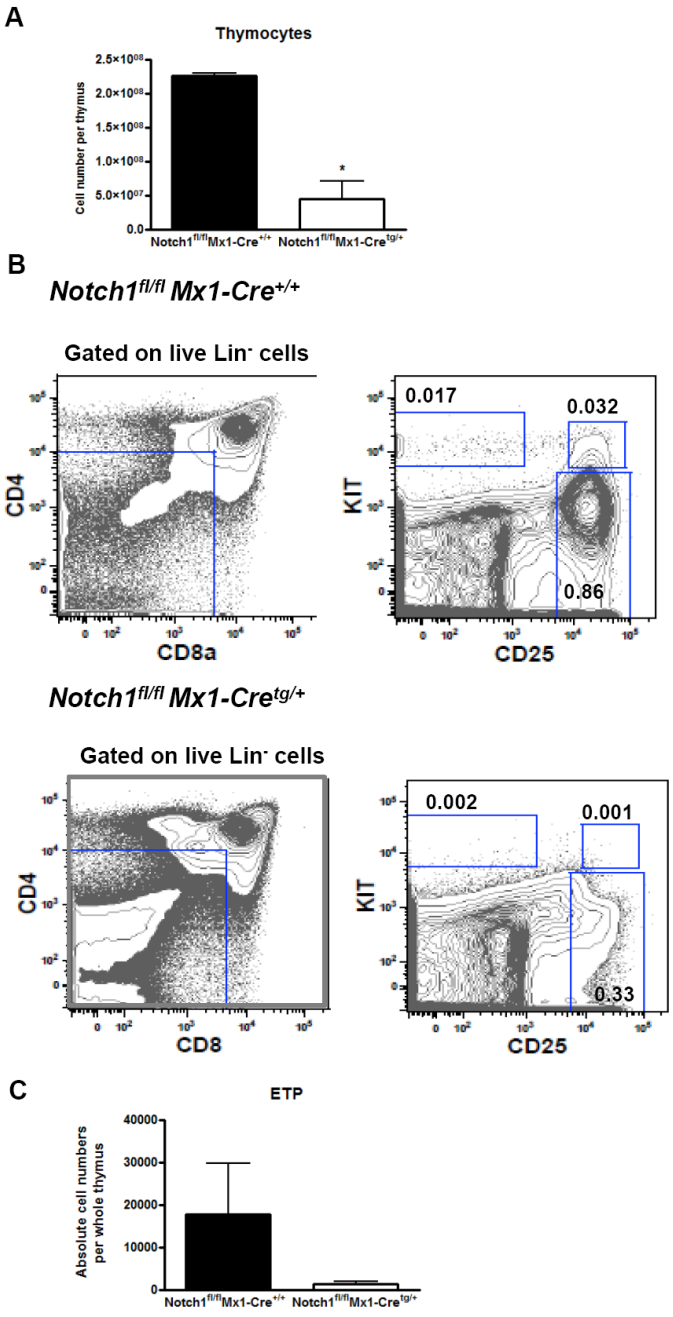


Figure 3.2. NOTCH1 is indispensable for ETP generation and downstream T cell development in *Mx1Cre*-induced *Notch1*-deficient mice

Three-to-six days old *Notch1 Mx1-Cre* whole litter was treated with a single dose of poly(I:C). Mice were genotyped and analysed six-to-seven weeks after treatment.

(A) Mean (SEM) thymus cellularity (N=2 per genotype).

(B) $Lin^-B220^-CD4^-CD8^-CD25^-c\text{-}KIT^{hi}$ ETP frequencies were determined. Representative FACS profiles of thymic populations from treated *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ and *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ genotypes are shown. Mean (SEM) frequency values in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ (N=2) and control *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ (N=2) within indicated gate.

(C) Mean (SEM) absolute cell numbers of ETPs in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ (N=2) and control *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{+/+}$ (N=2) poly(I:C) treated mice.

For all panels * $p<0.05$, otherwise no significant differences between the *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ and control groups were observed.

3.2.3 Complete abrogation of Notch signaling results in a block in ETP generation in the thymus

Previously reported identical phenotypes between *Notch1*- and *Rbp-Jk*-deficient mice (Han et al., 2002; Krebs et al., 2003; Radtke et al., 1999; Saito et al., 2003) indicate that NOTCH1 is the critical receptor in thymic T cell development (Han et al., 2002; Krebs et al., 2003; Saito et al., 2003). As a result of the new evidence reported here for the largely dispensable role of NOTCH1 in the generation of ETPs, I next evaluated whether combined canonical Notch signaling plays a more essential role in the establishment of ETPs in the thymus, using the Vav-Cre system.

In canonical Notch signaling, signals from all four Notch receptors converge on the transcription factor RBP-Jk in the cell nucleus triggering Notch target gene activation (Lai, 2004) and therefore canonical Notch signaling is totally abrogated in the absence of *Rbp-Jk*. Thus, *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$

control mice were studied. At 7 weeks of age the thymic T cell subsets were analyzed by FACS. Notably, the ETP phenotype of *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice was markedly different from *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice. In fact, ETPs were virtually absent in *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ recombined mice (Figure 3.3A). Similar to what has been observed in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice but more severely in the *Rbp-Jk*-deficient mice, downstream T cell progenitors c-KIT^{+} DN2 and DN3 (Figure 3.3A), as well as maturing CD4SP, CD8SP and CD4^{+}CD8^{+} DP T cells (Figure 3.3B), were reduced in *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice compared to controls.

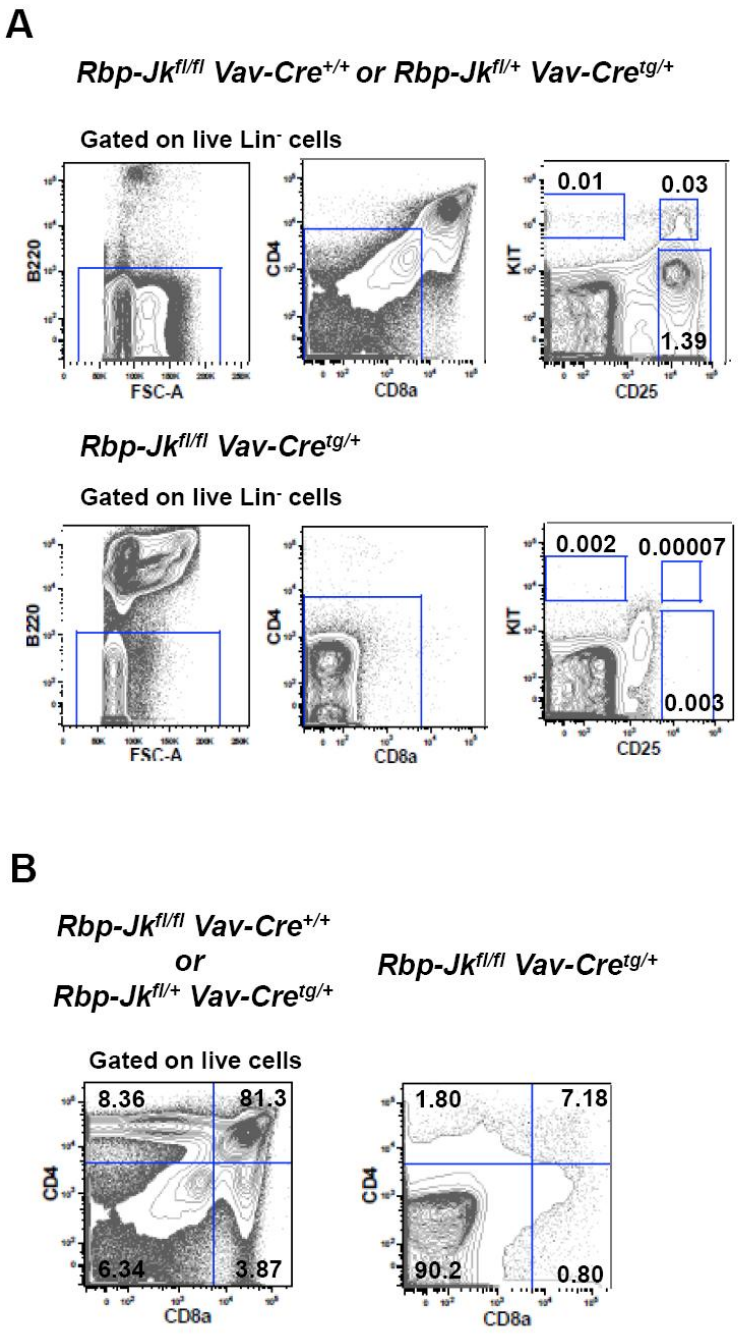


Figure 3.3. Complete disruption of Notch signaling hinders ETP generation in the thymus

(A) Seven weeks old *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=5) and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ (N=3) control mice were analysed and ETP, c-KIT^{+} DN2 and DN3 frequencies were determined by

FACS. Representative FACS profiles with mean frequencies from *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ genotypes.

(B) Representative CD4/CD8 FACS profiles from *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ (N=5) and *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{+/+}$(N=3) genotypes are shown. Numbers within gates indicate mean frequencies of CD4 SP, CD8 SP, DP and DN cells, respectively.

In conclusion, data reported in this chapter suggest that NOTCH1 is not essential for the establishment of ETPs in the thymus but critical for the transition from DN1 to DN2 stage. However, no ETPs are generated if canonical Notch signaling is completely disrupted, implicating that this signaling pathway is essential for early thymus T cell development. Furthermore, as several key Notch target genes are downregulated in *Notch1*-deficient ETPs, their upregulation in ETPs might be critical for the transition to DN2 and subsequent stages.

3.3 Discussion

Cre transgenic mice are widely used to delete a gene flanked by *loxP* sites in specific somatic tissues and at specific times, allowing its function to be studied (Branda and Dymecki, 2004; Furuta and Behringer, 2005).

Studies in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice implicated NOTCH1 to be of crucial importance for ETPs (Radtke et al., 1999). In contrast, it is here demonstrated using *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice that in fact NOTCH1 is largely dispensable for ETP establishment in the thymus.

Mx1-Cre is a non-lineage specific deleter, and although the levels of induced deletion are variable, it can promote gene inactivation in the liver, spleen, thymus, BM and lungs among many other tissues (Kuhn et al., 1995; Radtke et al., 1999). It is well known that NOTCH1 is involved in processes such as cell fate determination and survival in a variety of tissues (Lai, 2004), and therefore it cannot be disregarded that, in *Mx1-Cre*-mediated *Notch1* deletion, other non-haematopoietic tissues may be affected, influencing on the phenotype observed. In fact, it has been previously reported that *Notch1*$^{fl/fl}$*Mx1-Cre*$^{tg/+}$ poly(I:C) treated mice show a growth retardation (Radtke et al., 1999) which is not observed in the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice used herein. This may be due to the inducer treatment that is affecting the development of the *Notch1*-deficient mice, which may be more sensitive than the controls. Furthermore, there are important limitations associated with Cre-mediated recombination induced by anti-inflammatory agents, such as IFN-α and poly(I:C) (Kuhn et al., 1995). First of all, it cannot be ruled out that thymocytes in *Notch1*-deficient mice are more sensitive to the poly(I:C) treatment than control mice, compromising cell survival, which may explain why ETPs are so much more dramatically affected in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ than *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$mice. In addition, it has been shown that poly(I:C) can stimulate the production of cytokines, other than endogenous interferon, and interfere with their signaling pathways (Kuhn et al., 1995). Indeed, although detected in control treated mice, no c-KIT^{+} cells are detected in *the Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ cells. It is reasonable to consider that thymic c-KIT^{+} cells, that include ETPs, in the *Notch1*-deficient mice, are more sensitive to poly(I:C) side effects than controls and therefore can downregulate c-KIT receptor, hindering the possibility of detecting c-KIT receptor

expressing cells. Furthermore, it is possible that other cytokine signaling pathways are affected by the treatment, affecting the population dependent upon these pathways. Some haematopoietic populations are dependent on cytokine signaling pathways, such as FLT3 and IL-7Rα pathways, namely LMPPs and ETPs, which may be affected by the interferon treatment (Adolfsson et al., 2005; Sambandam et al., 2005). Moreover, *Mx1-Cre* mice are sensitive to endogenous interferon, which may trigger Cre-recombinase activity without inducer treatment, and influence the phenotype observed (Furuta and Behringer, 2005; Kuhn et al., 1995). Finally, it has previously been shown that although liver and kidney did not show any histological abnormalities, and B and T cell frequencies in the spleen were normal, T cells in the thymus, known to be sensitive to stress, were reduced by about 50% in poly(I:C)-treated *Mx1-Cre*$^{tg/+}$ mice without any other genetic modifications (Kuhn et al., 1995). Thus, an *Mx1-Cre* inducible system should be used with caution when studying T cell development.

Vav-Cre mediated pan-haematopoietic specific recombination is activated in mice from around day e10 onwards (Chen et al., 2009; Georgiades et al., 2002) and after *Notch1* dependent HSC generation has occurred (Kumano et al., 2003). Thus, *Notch1* gene function can be studied from embryonic stages throughout adult life. This represents a great advantage of the *Vav-Cre* system compared to *Mx1-Cre*, as it allows the study of T cell development from early stages of development, namely in embryonic and neonatal mice, when T cell development takes place and the frequency of progenitors is greater than in older mice (Ceredig et al., 2007; Hollander et al., 2006). Furthermore, all the side effects from poly(I:C) administration observed in the *Mx1-Cre*-mediated recombination are avoided when

using the *Vav-Cre* system and side effects from deletion of *Notch1* in other tissues than the endothelial and haematopoietic are circumvented. Hence, *Vav-Cre* would be a better and more feasible Cre system than *Mx1-Cre* to be used in the study of early T cell development in neonates.

It is here demonstrated that *Notch1* is not required for the generation of ETPs. In addition, the presence of ETPs in *Notch1*-deficient mice supports the view that NOTCH1 is not important for homing to the thymus by multipotent progenitors from the BM. Maillard and colleagues demonstrated that canonical Notch signaling is dispensable for BM HSC function and maintenance (Maillard et al., 2008). Furthermore, BM LSKs are still detectable in the blood of *dnMAML1* conditional knock-in mice (Sambandam et al., 2005). These studies imply NOTCH1 to be not necessary for the maintenance of progenitors in the BM and their migration in the blood. However, it remains to be established where Notch signaling is first activated, if in the BM, where *Jagged1* ligand is highly expressed (Lehar et al., 2005), in the blood while multipotent progenitors travel to the thymus or early after entry of the TSP into the thymus. Importantly, Notch signaling has been suggested to be activated in lymphoid progenitors only after contact with thymic epithelium (Harman et al., 2003), further supporting the view that *Notch1* is dispensable for thymus homing by BM progenitors, but crucial after progenitors have seeded this organ. Since deletion in *Vav-Cre* mice starts very early in the embryo, it cannot be excluded that maybe NOTCH2 is able to compensate from early stages for the absence of NOTCH1, a redundancy event that may be missed in *Notch1 Mx1-Cre* mice, and that can explain the different thymic phenotypes observed between the two mouse strains.

Previous reports have shown that Notch signaling is crucial for the transition of DN3 to DN4 stages (Tanigaki et al., 2004; Wolfer et al., 2001) as well as for DN3 β-selection and subsequent survival of the selected thymocytes (Ciofani and Zuniga-Pflucker, 2005; Wolfer et al., 2002). Here we further demonstrate that NOTCH1 is critical for the transition from DN1 to the DN2 progenitors. Accordingly, supporting the findings of studies where it has been demonstrated that upregulation of surface CD25 and downregulation of FLT3 in DN2 is *Notch1* dependent (Sambandam et al., 2005), this is further corroborated by Notch target gene expression data presented here.

Nevertheless, although the identified ETPs in the *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice are indeed phenotypic ETPs, expressing high levels of c-KIT receptor and the only thymic progenitors expressing *Flt3 receptor* in the thymus, a functional validation of these cells where their multipotency would be assessed, both *in vitro* and *in vivo*, would be mandatory to prove that they are *bona fide* ETPs.

It is not clear from the results above whether other Notch receptors in the canonical Notch signaling pathway may be involved in TSP homing or generation of ETPs. The fact that *Notch2*- (Saito et al., 2003) and *Notch3*-deficient mice (Krebs et al., 2003) have no reported T cell development abnormalities would strongly suggest that neither of these receptors are implicated in these events or alternatively that NOTCH1 might act as a compensatory factor in T cell development. However, and very strikingly, ETPs are virtually gone in *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ thymus, suggesting that a Notch receptor different from NOTCH1, or a factor from the Notch signaling pathway, may play an important role in thymus settling by a TSP coming from the BM. Alternatively, two or more receptors,

including NOTCH1, may act together to promote TSP thymus-seeding and/or establishment of ETPs, and in the absence of *Notch1*, its function is compensated by its partner. In fact, *Notch1*-deficient ETPs, cultured on a T cell promoting *in vitro* system use NOTCH2 to differentiate into T cell lineage committed cells (Benz et al., 2008; Besseyrias et al., 2007), implying a redundancy between Notch receptors in T cell development further suggested here *in vivo*. Nevertheless, data here do not show specific redundancy between NOTCH1 and NOTCH2. One way to address this question would be to analyze the thymus of *Notch1*$^{-/-}$ *Notch2*$^{-/-}$ double knockout mice.

In conclusion, data herein strongly suggest that Notch signaling is crucial for either thymus settling by a TSP from the BM or for the generation of ETPs, in a largely *Notch1*-independent manner, while *Notch1* is critical for the DN1 to DN2 transition. However, the place (intra or extrathymically) and identity of the first cell receiving Notch signals remains to be investigated. In addition, the role of Notch in the stepwise lineage restriction events from a multipotent progenitor coming from the BM to a T cell restricted progenitor in the thymus remains unresolved.

3.4 Future directions

Functional and molecular characterization of *Notch1*-deficient ETPs

Studies reported here demonstrate that ETPs do not depend upon NOTCH1 for their generation, in *Notch1*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice. However, these conclusions are based on phenotypic analysis and therefore functional characterization of these *Notch1*-deficient ETPs is required to establish these cells as *bona fide* ETPs.

Functionally, ETPs are multipotent progenitors, with prominent T cell potential but also B, myeloid, Nk and DC potential (Allman et al., 2003; Bell and Bhandoola, 2008; Benz and Bleul, 2005; Bhandoola et al., 2003; Sambandam et al., 2005; Wada et al., 2008). Phenotypic ETPs present in the *Notch1*-deficient mice should therefore be assessed for their multilineage potential, both *in vitro* and *in vivo*. The lineage multipotency of ETPs should be reflected in the expression of B, T and myeloid lineage-related genes, requiring a lineage priming analysis.

Evaluate the role of Notch signaling in thymus seeding by a TSP and its differentiation into ETPs

Another remaining question is the hierarchical proximity of the ETPs to a candidate thymus-seeding multipotent progenitor in the BM. Recent data from our laboratory have implicated LMPPs to be a TSP candidate closely related to ETPs, given their functional and molecular similarity (Luc S *et al*, 2011, manuscript submitted). However, these studies did not investigate how dependent on Notch signaling LMPP to ETP differentiation events are. Global gene profiling of *Notch1*- and *Rbp-Jk*-deficient ETPs and comparison to *Notch1*- and *Rbp-Jκ*-deficient BM candidate TSPs, particularly the lymphoid-directed progenitor, LMPP, as well as downstream thymic populations, such as DN2, will give insight into the hierarchical organization of transcriptional lineage programs between these populations.

Furthermore, the absence of ETPs in *Rbp-Jk*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice raises the question of whether canonical Notch signaling is essential for the migration of TSPs to the thymus. Can ETPs develop normally once they have overcome the migration step and reached the thymus? Intrathymic transplantation assays, where

Rbp-Jk-deficient TSPs would be intrathymically transferred into conditioned mice and their capacity to generate ETPs assessed would provide an answer to this question.

Also, it would be of great importance to identify and characterize the candidate BM TSPs in the *Rbp-Jk*-deficient mice and how they are affected in terms of numbers and function upon loss of *Rbp-Jk*. Additionally, the presence of candidate TSPs in the peripheral blood and their migration pathway to the thymus should be investigated, as progenitors going to the thymus should be detected in the blood. Finally, the migration properties of candidate TSPs should be assessed.

Study the role of Notch in the stepwise lineage restriction events from a multipotent progenitor to a T cell progenitor

Finally, the role of Notch signaling in the stepwise lineage restriction from a BM multilineage progenitor into a unipotent T cell progenitor remains to be understood. How are the different lineage potentials lost during T cell lineage restriction? What is the role of Notch in these lineage restriction events? Is the B cell potential lost prior to the myeloid potential? An assay where Notch signaling exposure and activation on TSPs and ETPs could be controlled would bring insight into these lineage commitment events and their dependence on Notch signals.

4

GATA3 is dispensable for haematopoietic stem cell regulation

4.1 Introduction

GATA transcription factors GATA1-3 are expressed in the haematopoietic system (Ho et al., 2009). While GATA1 has been implicated in the regulation of committed erythrocytes, megakaryocytes and eosinophils (Fujiwara et al., 1996; Pevny et al., 1991; Shivdasani et al., 1997; Yu et al., 2002), GATA2 acts earlier in the hierarchy and has been shown to be important for proliferation and survival of HSCs (Ling et al., 2004; Rodrigues et al., 2005; Tsai et al., 1994; Tsai and Orkin, 1997). GATA3 is essential for T cell development and is best known as the master regulator of Th2 cell differentiation (Ho et al., 2009; Zheng and Flavell, 1997; Zhu et al., 2004). However, its role in HSC regulation has not been extensively investigated. *Gata3* transcripts are detected in HSCs and downregulated in downstream MPPs (Benveniste et al., 2010; Lai and Kondo, 2007; Sambandam et al., 2005; Tydell et al., 2007), which would suggest a regulatory function of GATA3 in the HSC compartment. Moreover, it has been recently shown that foetal liver *Gata3* null and wild type donor cells contributed at comparable levels to $Gr1^+Mac1^+$ myeloid and $B220^+$ B cell reconstitution in the blood of short-term transplanted mice (Hosoya et al., 2009). Although these studies may suggest GATA3 to be dispensable in the HSC compartment, long-term reconstitution capacity as well as self-renewal potential of *Gata3*-deficient HSCs was not assessed (Hosoya et al., 2009). Moreover, detailed phenotypic analysis of the HSC compartment upon loss of *Gata3* has not been carried out so far. Thus, a study of the phenotypic and self-renewal potential of HSCs lacking *Gata3* is required to establish whether GATA3 plays a role in these cells.

4.2 Results

4.2.1 HSC maintenance is unaffected by the loss of *Gata3*

Gata3 has been shown to be expressed at higher levels in HSCs than in MPPs (Benveniste et al., 2010; Lai and Kondo, 2007; Sambandam et al., 2005; Tydell et al., 2007), suggesting a differential role of GATA3 in the HSC compartment. The mRNA expression levels of *Gata3* were assessed in highly purified long-term LSKFLT3$^-$CD48$^-$CD150$^+$ HSCs and compared to TSP-containing LMPPs as well as LIN$^-$CD25$^-$KIT^{hi}FLT3$^+$ ETPs, known to express increased levels of *Gata3* transcript (Lai and Kondo, 2007; Sambandam et al., 2005). As previously suggested by others, *Gata3* is readily detected in long-term HSCs and is downregulated in LMPPs before increasing again in the thymic ETPs (Figure 4.1A).

Gata3 is embryonically lethal beyond day e11 due to noradrenaline deficiency that leads to cardiac dysfunction (Lim et al., 2000; Pandolfi et al., 1995). Zhu and colleagues generated a conditional *Gata3* mutant using the *Cre-loxP* system (Zhu et al., 2004), by flanking exon 4 in the *Gata3* gene with two *loxP* sites. Importantly, deletion of exon 4 prevents the expression of exon 5 and more distal exons (Zhu et al., 2004). Thus, in order to overcome lethality and so allow studies of the role of GATA3 in HSC regulation to take place, *Gata3*$^{fl/fl}$ mice were crossed to *Vav-Cre*$^{tg/+}$ transgenic mice (de Boer et al., 2003), resulting in viable offspring in which recombination through *Vav* promoter occurs shortly after the emergence of HSCs from the haemogenic endothelium (Chen et al., 2009). Phenotypic FACS

analysis was then pursued to evaluate how the absence of *Gata3* affects the size of the HSC compartment. First, recombination efficiency in LSK haematopoietic stem cells under the *Vav* promoter was investigated in young adult *R26R*$^{YFP/+}$ *Vav-Cre*$^{tg/+}$ and *R26R*$^{YFP/+}$ *Vav-Cre*$^{tg/+}$ control mice. As shown in figure 2.1, virtually all LSK cells expressed YFP showing highly efficient *Vav-Cre*-mediated recombination (Figure 2.1). BM cellularity was identical between 1 week old *Gata3*-deficient and control mice (Figure 4.1B). Phenotypically defined long-term HSCs were not affected by the loss of *Gata3*, as shown by similar frequencies in both *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ control mice (Figure 4.2C and 4.2D), suggesting that *Gata3* is dispensable for steady-state maintenance of HSCs in the BM. Deletion efficiency of *Gata3*-deficient HSC was next evaluated and, as shown in figure 4.1F, Vav-Cre mediated recombination of *Gata3* was highly efficient in these primitive cells (Figure 4.1E).

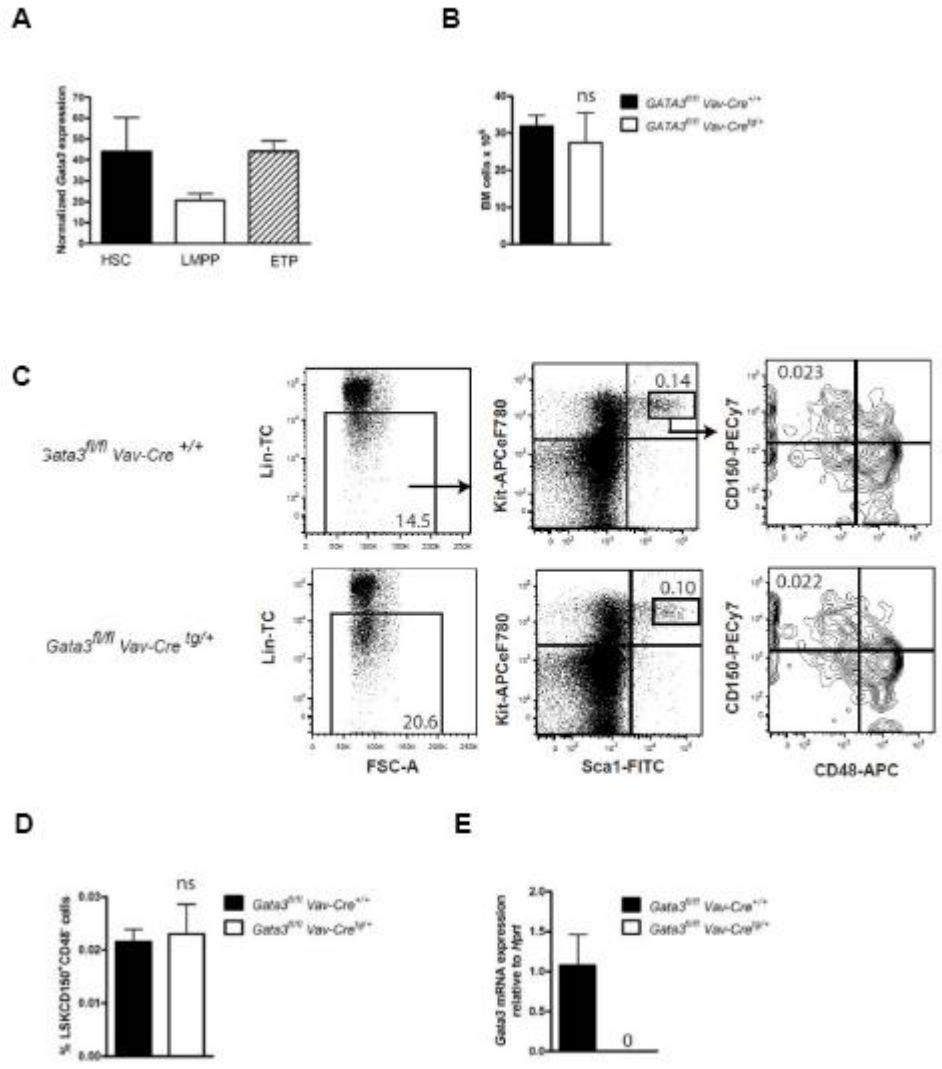


Figure 4.1. *Gata3* is dispensable for steady state HSC and multipotent progenitor maintenance in the BM

(A) *Gata3* mRNA expression in wild type LSKFLT3^{-}CD150^{+}CD48^{-} long-term HSCs, LSKFLT3^{hi} (LMPP) BM cells and Lin^{-}CD44^{-}CD25^{-}KIT^{+}FLT3^{+} (ETP) thymocytes was assessed. Data expressed as mean (SEM) normalized Robust Multi-array Averages. N=3 experiments.

(B) Bone marrow cellularity in 1 week old *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* and *Gata3$^{fl/fl}$ Vav-Cre$^{+/+}$* mice. Data expressed as mean (SD), N=3-4 mice per genotype. ns: non-significant.

(C) Representative FACS profiles for LSKCD150^{+}CD48^{-} cells isolated from 1 week old mice. Numbers indicate percentage of total BM cells within the indicated gate. N=3-4 mice per genotype.

(D) Mean (SD) frequency of LSKCD150^{+}CD48^{-} cells. N=3-4 mice per genotype. ns: non-significant.

(E) LSKCD150^{+}CD48^{-} cells were isolated from 1-2 week old *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice or *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ and subjected to quantitative gene expression analysis for the *Gata3* gene using a dynamic array (Biomark Fluidigm). Mean (SEM) *Gata3* mRNA expression level normalized to *Hprt*. A total of 100 cells per well were sorted per genotype (2-3 wells per genotype).

4.2.2 Gata3 is dispensable for maintenance and self-renewal of HSCs

Previous studies have implicated GATA3 as not necessary for myeloid and B lymphoid regeneration, while T cell emergence was impaired in the absence of *Gata3* (Hosoya et al., 2009; Ting et al., 1996). However, the long-term reconstitution capability of *Gata3*-deficient HSCs has never been investigated. Thus, functional assays were performed to investigate whether GATA3 regulates expansion and self-renewal of HSCs. Lethally irradiated recipients were competitively transplanted with *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ or *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ control BM cells along with equal amounts of competitor wild type cells. Peripheral blood was analyzed by FACS 4-6 months after transplantation for multilineage reconstitution. Myeloid (GR-1^{+} MAC-1^{+}) and B (CD19^{+}) cell reconstitution was comparable between the two groups (Figure 4.2A and 4.2B). T cells were absent in

mice transplanted with *Gata3*-deficient BM cells in contrast to controls, further confirming sufficient *Gata3* ablation in BM cells of *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice (Figure 4.2A and 4.2B). Similarly, normal myeloid and B cell reconstitution was observed 11 months after transplantation in mice transplanted with *Gata3*-deficient BM cells (Figure 4.3C).

Self-renewal capacity of HSCs lacking *Gata3* was next assessed by performing secondary transplantation experiments. Mice were analyzed 8 months after transplantation, and while no *Gata3 null* T cells were detected, myeloid and B cells were reconstituted at similar levels as non-deleted donor cells (Figure 4.3D).

Taken together, these data demonstrate that GATA3 has no essential regulatory function in HSCs and is therefore dispensable for their steady-state maintenance and self-renewal.

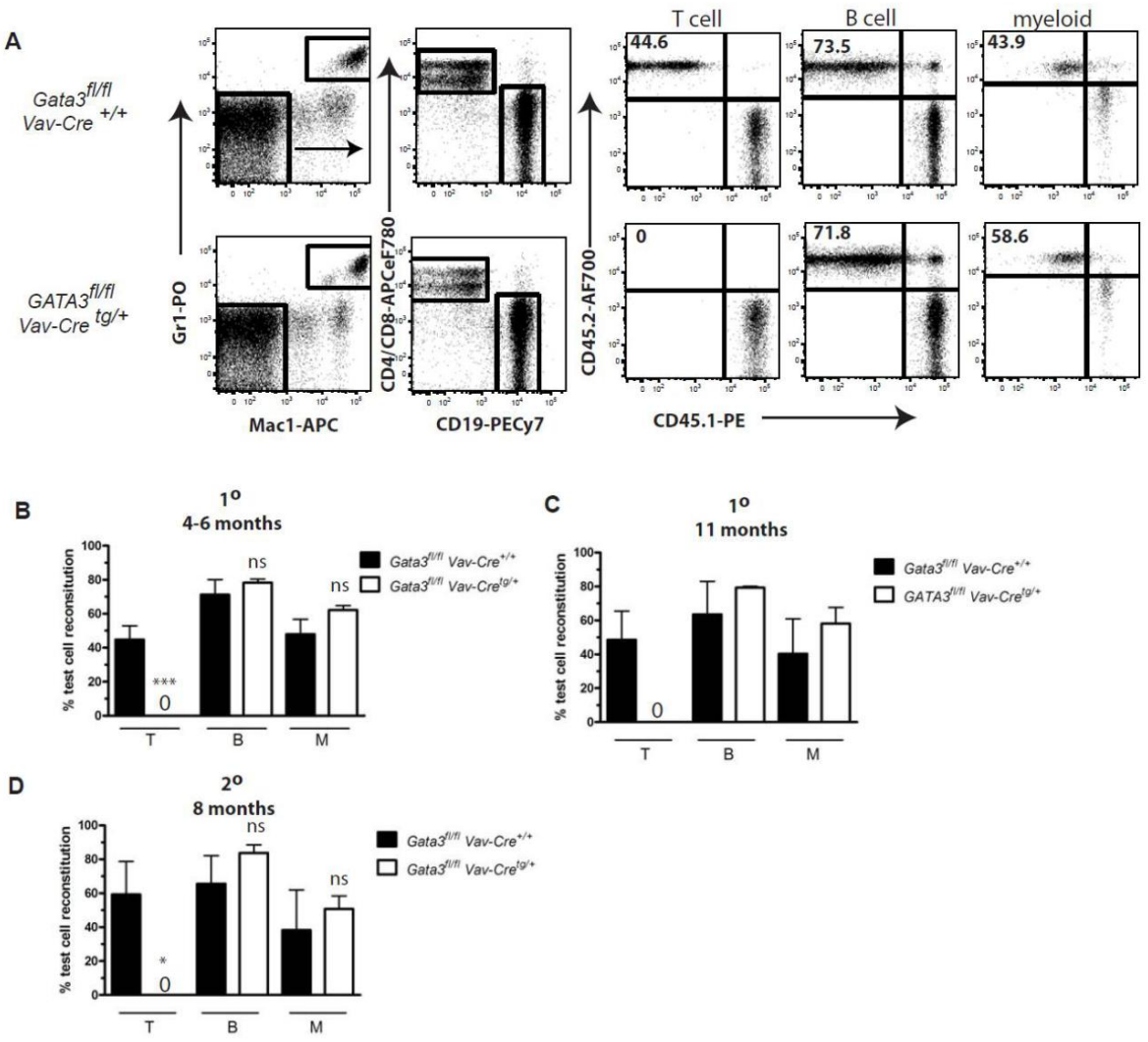


Figure 4.2. *Gata3* is redundant for HSC expansion and self-renewal

Wild type CD45.1 recipient mice were lethally irradiated and transplanted with 0.5-2 million CD45.2 BM cell from either 1-2 weeks old *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ or *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ control mice along with similar dose of competitor WT CD45.1 BM cells. Peripheral blood analysis was performed **(A, B)** 4-6 or **(C)** 11 months after transplantation.

(A) Representative FACS profiles of peripheral blood mutilineage reconstitution analysis 4-6 months after transplantation. Numbers indicate percentage of indicated gate within reconstituted T (CD4$^+$/CD8$^+$), B (CD19$^+$) and myeloid (Gr1$^+$Mac1$^+$) cells. N=3-6 mice per group, 2 experiments.

(B-C) Mean percentage (SEM) of test cell ($CD45^+$) reconstitution within the T, B and myeloid cell lineages, respectively, **(B)** 4-6 or **(C)** 11 months after transplantation (B: N= 6-9 mice per group, 2 experiments; C: 3 mice per group, 1 experiment) . 1°: primary reconstitution.

(D) Eight months after transplantation half femur equivalents from primary recipients were transplanted into secondary wild type (CD45.1) recipients. Data show mean (SEM) percentage of test cell reconstitution of T, B and myeloid total blood cells. N=3-6 mice per group, 1 experiment. 2°: secondary reconstitution.

*p< 0.05, ***p< 0.001, ns indicates non-significant (in panel C significance levels were not established due to limited numbers of mice investigated).

4.3 Discussion

Both GATA1 and GATA2 factors intervene in the regulation of haematopoiesis in the BM at different stages (Fujiwara et al., 1996; Ling et al., 2004; Pevny et al., 1991; Rodrigues et al., 2005; Shivdasani et al., 1997; Tsai et al., 1994; Tsai and Orkin, 1997; Yu et al., 2002). GATA3 is also found to be expressed in the haematopoietic system. However, although essential for T cell development in the thymus (Ho et al., 2009), a role for GATA3 in BM HSC maintenance, expansion and self-renewal remains to be established. It has been reported that *Gata3 null* cells can normally reconstitute myeloid and B cell compartments in the BM (Hosoya et al., 2009; Ting et al., 1996). However, in these studies only short-term reconstitution potential has been assessed. In fact, to date no studies have reported the role of GATA3 in the HSC defining properties for

long-term reconstitution and self-renewal. Furthermore, how absence of *Gata3* affects the numbers of long-term highly purified HSCs has never been investigated. A very stringent and complete analysis of the stem cell compartment at the phenotypic and functional levels was performed and irrefutably shows that GATA3 is dispensable for HSC maintenance in both steady-state and under conditions of great stress, such as after transplantation, where HSCs are required to cycle and expand (Verfaillie, 2002; Wilson et al., 2008). Moreover, HSC self-renewal capacity is preserved upon loss of *Gata3* as assessed by secondary transplantation experiments. Redundancy between GATA factors has been previously demonstrated in haematopoiesis (Chen and Zhang, 2001). In fact, GATA2 has been shown to be important for regulation of HSCs, therefore it cannot be excluded that *Gata2* is compensating for the loss of *Gata3* in regulating HSCs.

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5

Role of GATA3 in early T cell commitment

5.2 Introduction

ETPs are the most immature T cell progenitors found in the thymus (Allman et al., 2003) and their establishment is dependent upon activity of transcriptional regulators acting in the BM and/or in the thymus. Canonical Notch signaling through RBP-Jk is crucial for the emergence of ETPs in the thymus (chapter 3). However, NOTCH1 receptor is dispensable for the establishment of these progenitors (Chapter 3) although critical for T cell development (Pui et al., 1999; Radtke et al., 2010; Radtke et al., 1999; Wilson et al., 2001). Similarly, GATA3 is essential for the later stages of T cell development (Ho et al., 2009; Pai et al., 2003; Zheng and Flavell, 1997; Zhu et al., 2004) and has been shown to be a direct Notch target gene in Th2 cells (Amsen et al., 2007; Fang et al., 2007). Nevertheless, it is not known whether GATA3 has a regulatory role in the stage and lineage specific generation of T cells from early stages and whether Notch and GATA3 interact in early T cell commitment events.

T cell production in the thymus is secured by continuous replenishment of thymus settling progenitors coming from the BM (Goldschneider et al., 1986; Scollay et al., 1986). Progenitors in the BM such as HSCs, CLPs or LMPPs have been suggested as seeding the thymus and generating ETPs (Allman et al., 2003; Krueger and von Boehmer, 2007; Martin et al., 2003; Schwarz et al., 2007). Accordingly, the phenotypic and functional similarities between LMPPs and ETPs, pinpoint the former as a potential immediate predecessor of ETPs in the BM (Adolfsson et al., 2005; Lai and Kondo, 2007; Schwarz and Bhandoola, 2004; Schwarz et al., 2007). However, and of interest, is that *Gata3* is expressed at

higher levels in HSCs than LMPPs and increase again in ETPs (chapter 4 and (Lai and Kondo, 2007; Sambandam et al., 2005; Tan et al., 2005), suggesting a potential role of GATA3 in setting up T cell development already at the stem cell stage.

Thus, I sought to study the role of GATA3 in the emergence of ETPs and whether its activity starts already in the thymus settling progenitor candidate, LMPP, in the BM.

5.2 Results

5.2.1 GATA3 is essential for the establishment of ETPs in the thymus

First, the ETP phenotype in the *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ newborn mice was assessed. *Gata3*$^{fl/fl}$ mice were bred to *Vav-Cre*$^{tg/+}$ transgenics and *Gata3*$^{fl/fl}$*Vav-Cre*$^{tg/+}$ generated. No gross abnormalities were observed in 8 days old *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice (data not shown). However, thymi of *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice were dramatically smaller than controls, which is reflected in a 104-fold reduction in thymus cellularity compared to controls (Figure 5.1A). Strikingly, ETPs were hardly found in *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice compared to controls (Figure 5.1B and 5.1C), and consequently downstream DN2 and DN3 progenitor subsets were severely reduced as well (Figure 5.1C and 5.1D). Notably, FACS analysis consistently showed that *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ thymocytes could hardly make it to the more mature CD4/CD8 stages, as shown by the severely reduced frequencies of CD4 SP, CD8 SP and CD4^{+}CD8^{+} DP cells (Figure 5.1E). These data imply that *Gata3* is

critical for ETPs in the thymus, consequently arresting T cell differentiation in the downstream stages.

To evaluate whether the few remaining ETPs in *Gata3*-deficient mice indeed lacked *Gata3* expression, *Gata3* mRNA was evaluated in ETPs from *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and compared to controls. As shown in Figure 5.1F, the *Gata3* gene was inactivated by virtually 100% in purified ETPs, revalidating the reliability of using *Vav-Cre* transgenics in the study of gene functions in the haematopoietic system.

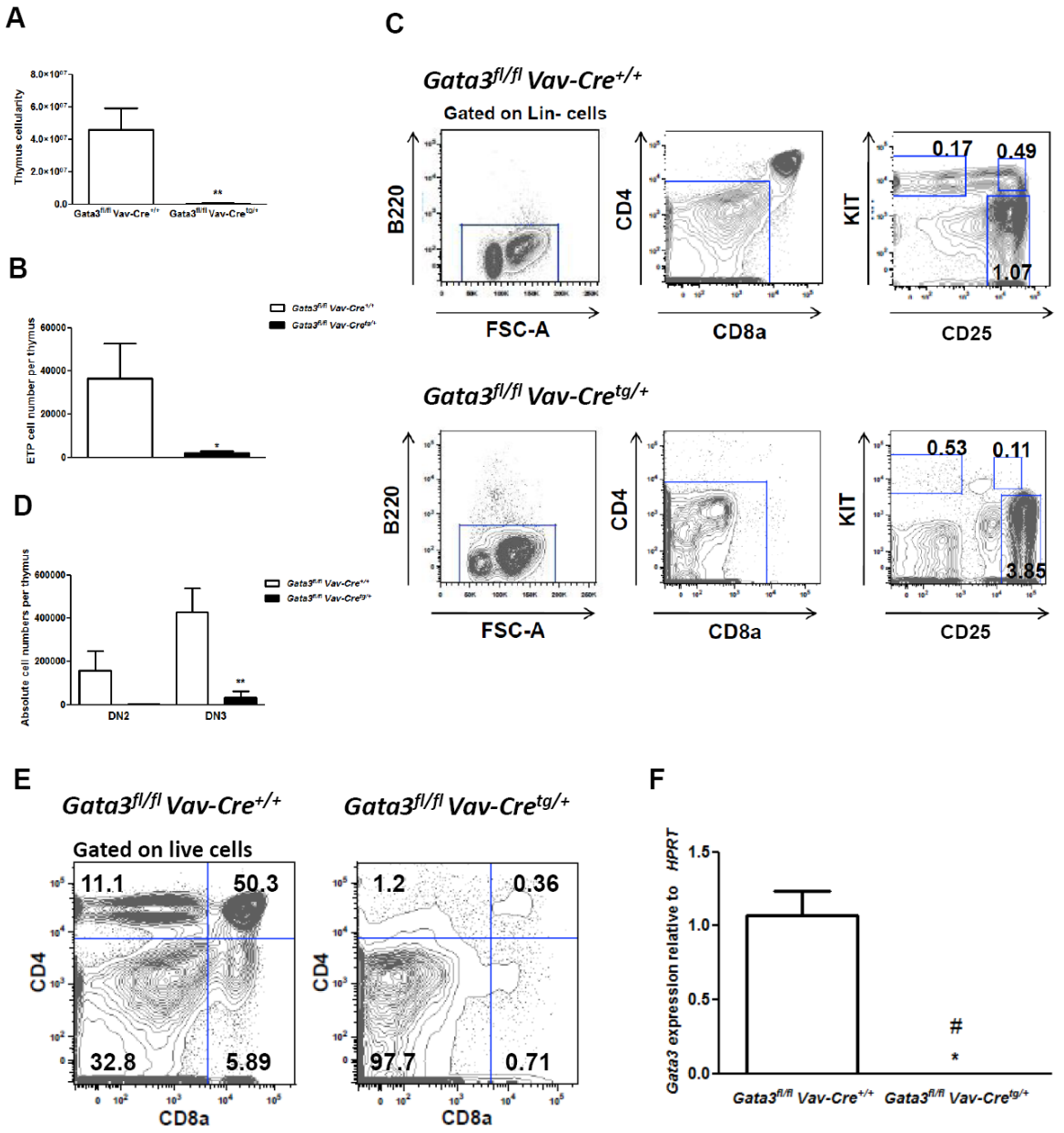


Figure 5.1. GATA3 is essential for the generation of ETPs

(A) Thymi from newborn up to 8 days old *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice (N=11) and littermate controls *Gata3*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ or *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ (N=11) were harvested and analyzed by FACS. Mean (SEM) thymus cellularity is shown.

(B) The absolute numbers of Lin^{-}CD4^{-}CD8^{-}CD25^{-}c-KIT^{hi} ETPs were determined in the thymus of the same mice. Graph plots shown mean (SEM) values.

(C) Representative FACS profiles show mean percentages of ETP, c-KIT$^+$ DN2 and DN3 cells from *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* (N=11) and *Gata3$^{fl/+}$ Vav-Cre$^{tg/+}$* or *Gata3$^{fl/fl}$ Vav-Cre$^{+/+}$* (N=11) control mice. Numbers indicate percentage within indicated gate.

(D) Mean (SEM) absolute numbers of DN2 (Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^+$c-KIT$^+$) and DN3 (Lin$^-$B220$^-$CD4$^-$CD8$^-$CD25$^-$c-KIT$^-$) progenitor subsets in the *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* (N=9 and N=6 in DN2 and DN3, respectively) and control mice ((N=10 and N=7 in DN2 and DN3, respectively).

(E) The proportion of CD4 SP, CD8 SP, CD4$^+$CD8$^+$ DP and CD4$^-$CD8$^-$ DN were evaluated in *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* and *Gata3$^{fl/+}$ Vav-Cre$^{tg/+}$* or *Gata3$^{fl/fl}$ Vav-Cre$^{+/+}$* control mice by FACS. Representative profiles for each genotype are shown. Numbers indicate representative mean percentages of CD4/CD8 populations.

(F) ETPs were sorted from individual *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* (N=1) and control *Gata3$^{fl/fl}$ Vav-Cre$^{+/+}$* (N=2) mice and expression of *Gata3* analysed using the Fluidigm Assay. Gene expression values were normalized to *Hprt*. For all experiments, 2-3 replicates per cell population were used for each individual mouse in both *Gata3*-deficient and control groups. Data show mean (SEM) values of all replicates for each cell population. Samples in which the mean value of replicates was ≤0.001 (relative to *Hprt* expression), were considered to be below cut-off value (#).

For all panels * $p<0.05$, ** $p<0.01$, otherwise no significant differences between *Gata3$^{fl/fl}$ Vav-Cre$^{tg/+}$* and the control groups were observed.

5.2.2 Multipotent progenitors in the BM are not affected by the loss of *Gata3*

Multipotent progenitors in the BM are thought to receive signals that instruct them to migrate via the blood and seed the thymus. However, the first cell settling the thymus remains to be identified. ETPs are the earliest progenitors identified to date in the thymus (Allman et al., 2003). These progenitors are phenotypically and functionally very close to LMPPs, and therefore the hypothesis that LMPP is the predecessor of ETP has been considered (Adolfsson et al., 2005; Allman et al., 2003; Balciunaite et al., 2005; Porritt et al., 2004). Nevertheless, earlier multipotent progenitors, including HSCs, cannot be ruled out as the TSPs entering the thymus.

Thus, the effect of *Gata3* deletion in the size of the MPP-containing LSK population as well as LMPPs was investigated in *Gata3*-deficient mice. As shown in Figure 5.2, the numbers of both populations were unaffected in these mice. These data, in addition to an unaffected HSC compartment in *Gata3* null mice (chapter 4) suggest that GATA3 is not important for migration of TSPs to the thymus but is rather critical after TSPs have seeded this organ, most likely acting in the earliest T cell commitment events.

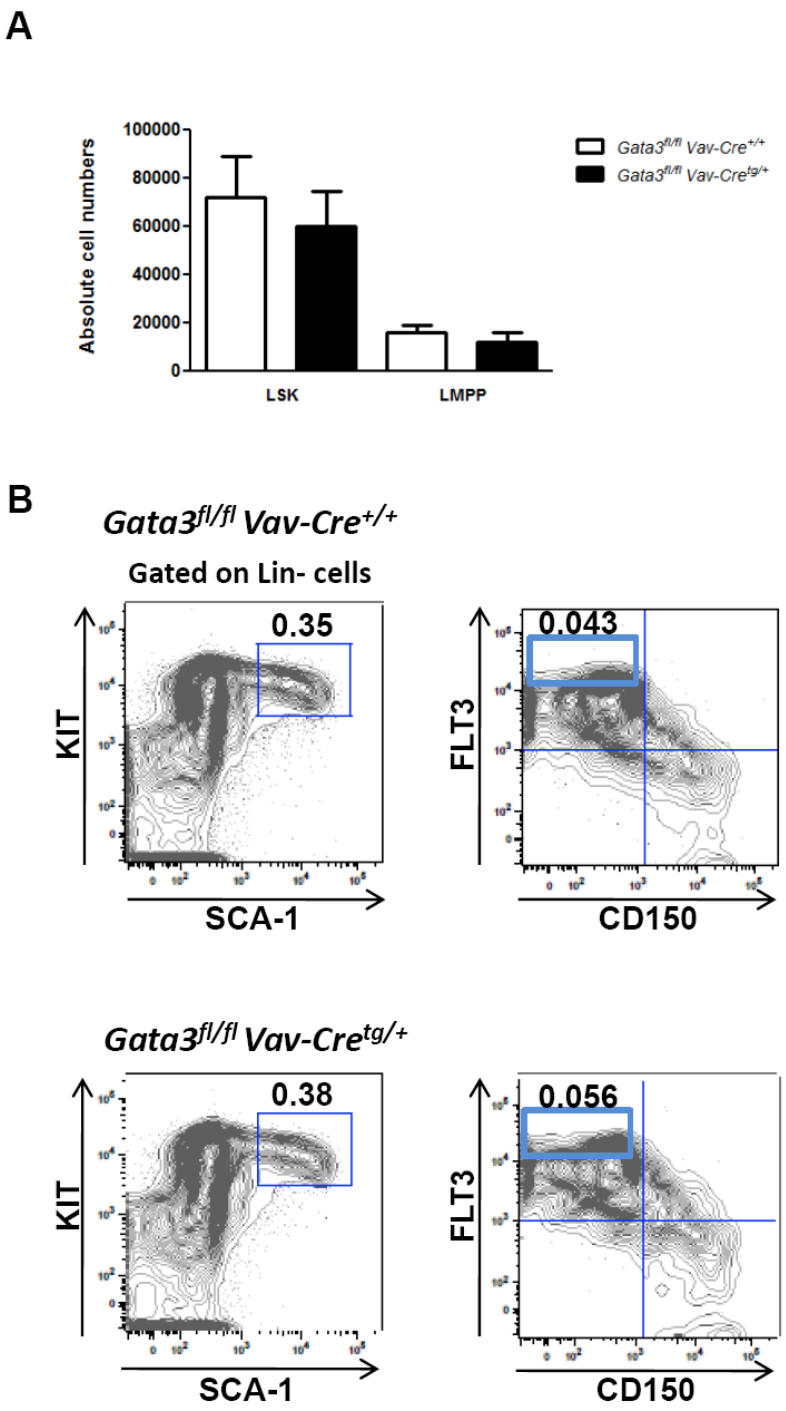


Figure 5.2. Multipoptent progenitors in the BM are not affected by the loss of *Gata3*

(A) BM from newborn-to-8 days old *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ mice (N=6) and littermate *Gata3*$^{fl/+}$ *Vav-Cre*$^{tg/+}$ or *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ (N=7) control mice was analyzed. Mean (SEM) values of

absolute cell numbers of LSKs and Lin$^-$Sca-1$^+$c-KIT$^+$Flt3^{hi} LMPPs are shown. No significant differences between *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and the control groups were observed.

(B) Representative BM FACS profiles with mean frequencies of LSK and LMPP subsets for each *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ and *Gata3*$^{fl/fl}$ *Vav-Cre*$^{+/+}$ genotypes are shown. Numbers indicate percentage within indicated gate.

5.3 Discussion

Although established to be essential for T cell development from the DN3 stage (Ho et al., 1991; Pai et al., 2003; Zhu et al., 2004), the role of GATA3 in early thymopoiesis is unclear. Suppression of GATA3 results in an arrest of Thy1$^+$ T cells (Anderson et al., 2002; Chen and Zhang, 2001; Taghon et al., 2001; Taghon et al., 2007). Furthermore, mRNA levels of *Gata3* augment significantly from BM progenitors to ETPs (Lai and Kondo, 2007; Sambandam et al., 2005), suggesting that GATA3 may be involved in early steps of T cell development.

Accordingly, studies reported here demonstrate that GATA3 is important for the establishment of ETPs in the thymus. In fact, while pursuing these studies, Engel´s laboratory published a study similarly showing that GATA3 is crucial for ETP emergence in foetal and adult mice (Hosoya et al., 2009). In these studies *Gata3* hypomorphic mutant embryos were used for the foetal studies and ETPs were less than 20% of the wild type controls, while *Gata3*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice were investigated in the adult studies where ETPs were hardly detected (Hosoya et al., 2009). Herein, *Gata3*$^{fl/fl}$ *Vav-Cre*$^{tg/+}$ neonates were used, where, as mentioned

before, *Gata3* recombination under the haematopoietic-specific *Vav* promoter takes places immediately after HSCs have been generated and therefore the effects of gene deletion may be studied early after birth, when the frequency of T cell progenitors is higher than in adults (Ceredig et al., 2007). Thus, in agreement with Hosoya *et al* studies, virtually no ETPs were found in *Gata3*-deficient neonates and consequently, downstream T cell stages were also severely reduced. Nevertheless, neither my nor Hosoya´s studies clarify where (in the BM or in the thymus) and in which progenitor GATA3 activity is first required.

The lack of ETPs in young *Gata3*-deficient mice may be due to a homing defect of the TSPs coming from the BM and settling the thymus to differentiate into ETPs or, alternatively, these BM progenitors might reach the thymus but are incapable of further differentiation into ETPs. In addition, *Gata3* may be required for survival and/or expansion of TSPs upon thymus seeding. Distinguishing between these distinct possibilities will require further studies.

Whether *Gata3* is active already in the BM, promoting T cell commitment has never been investigated. Although suggested to be the predecessor of ETPs (Adolfsson et al., 2005; Lai and Kondo, 2007; Schwarz and Bhandoola, 2004; Schwarz et al., 2007), *Gata3* expression in LMPPs is lower than in HSCs and ETPs (chapter 4; (Lai and Kondo, 2007; Sambandam et al., 2005)), suggesting that *Gata3* might be dispensable at the LMPP stage and rather promote ETP development directly from HSCs. However, HSCs have never been found in the thymus. Herein, it is reported that LMPP numbers, concomitantly with HSCs, are not affected by the loss of *Gata3*. However, how the absence of *Gata3* affected the migration and thymus homing of LMPPs was not investigated. Accordingly,

intrathymic transplantation experiments would provide insight into what happens once the migration step is overcome, whether the *Gata3*-deficient LMPPs could contribute to a normal ETP compartment and sustained T cell development. Furthermore, the capability of LMPPs to generate T cell progeny in the absence of *Gata3* could be tested in T cell promoting cultures *in vitro*.

Similarly, further studies are required to understand if, on the other hand, GATA3 rather regulates the TSP-ETP differentiation transition after the TSP has seeded the thymus. Furthermore, previous studies have suggested that levels of *Gata3* need to be very constrained otherwise cells enter apoptosis (Chen and Zhang, 2001; Hozumi et al., 2008b; Taghon et al., 2001; Taghon et al., 2007). Thus, it is possible that GATA3 regulates maintenance and survival of ETPs, however this hypothesis needs to be investigated. However, Hosoya and colleagues observed that GATA3 does not increase cell apoptosis nor interfere with cell-cycle progression in ETPs, thereby suggesting that ETP cell survival is not regulated by GATA3 (Hosoya et al., 2009).

Finally, GATA3 cannot promote T cell development in the absence of *Notch1 in vitro* (Hozumi et al., 2008b) and expression of both factors increases significantly in thymic progenitors compared to BM populations (Akashi et al., 2000; Lai and Kondo, 2007; Sambandam et al., 2005; Tydell et al., 2007; Zhong et al., 2005). Furthermore, *Gata3* has been shown to be a direct Notch target gene in Th2 cells (Amsen et al., 2007; Fang et al., 2007). These studies suggest that Notch signaling and GATA3 may interact to promote early T cell development. However, given that Notch signaling is higher in the thymus than in the BM (Lai and Kondo, 2007; Sambandam et al., 2005), if these two factors are related in early T cell

development then GATA3 activity would be important intrathymically rather than before TSPs reach the thymus. Nevertheless, a homing defect and impaired thymic ETP generation are not mutually exclusive.

Thus, all together, the main finding supported in studies reported here is that *Gata3* is critical for the establishment of ETPs in the thymus.

5.4 Future directions

Study the role of GATA3 in the migration of LMPPs to the thymus

As reported above, *Gata3*-deficient mice do not generate ETPs in the thymus which can be due to homing defects of the thymus settling progenitor traveling from the BM. In order to address this important question, intrathymic transplantation of LMPPs should be performed. If the establishment of ETPs is impaired only due to the incapability of LMPPs to migrate to the thymus, intrathymic transfer would allow overcoming this limitation and ETPs would be expected to be generated from *Gata3*-deficient LMPPs directly placed in the thymus.

Evaluate the role of Gata3 in early T cell commitment events in the embryo

Vav-Cre transgenics, that start recombining as early as day e10 in the embryo, are a powerful tool to investigate gene functions in the early T cell commitment events during late embryonic development. Therefore, it would be interesting to evaluate how *Gata3* loss affects foetal thymus seeding and subsequent generation of ETPs as well as downstream progenitors in foetal life.

Evaluate the relationship of Notch signaling and GATA3 in early T cell development

Although both NOTCH1 and GATA3 are very important for T cell development, it is demonstrated here that while NOTCH1 is dispensable for the emergence of ETPs (Chapter 3), GATA3 is essential for the establishment of these progenitors in the thymus. Furthermore, it has previously been shown that *Gata3* is directly regulated by Notch signaling in Th2 T cells, by binding of RBP-Jk to the upstream *Gata3* promoter (Amsen et al., 2007; Fang et al., 2007). In addition, it is well known that both GATA3 and NOTCH1 are critical for T cell development (Ho et al., 2009; Maillard et al., 2005), however, although it is clear that *Gata3* and Notch signaling directly intersect during Th2-cell development, even though *Notch1*-deficient mice (CD4-Cre) do not have a Th2 phenotype, it remains to be determined whether and how Notch signaling and GATA3 interact together in early T cell commitment events in the thymus.

Both *Notch1* and *Gata3* are upregulated from BM progenitors to thymic ETPs and DN subsets, and *Gata3* expression levels increase in uncommitted progenitors cultured in the presence of Notch signals and which commit towards the T cell lineage (Ho et al., 2009; Taghon et al., 2005). Furthermore, *Gata3* gene expression levels are downregulated in *Notch1*-deficient ETPs (Chapter 3). However, although these studies, together with the well established role of Notch in T cell development (Pui et al., 1999; Radtke et al., 1999), would strongly suggest that Notch signaling regulates *Gata3*, overexpression studies indicate that *Gata3*, in the absence of Notch is not capable of promoting T cell development, and in the

presence of Notch signaling, both *Gata3* overexpression or lack of it increase cell death. Thus, it would be an important step in the field to understand to what degree *Gata3* is dependent upon Notch and how Notch signaling and *Gata3* are related in the early T cell commitment events. Can overexpression of *Gata3* in BM TSPs or early T cell progenitors overcome the requirement for Notch signaling in the migration of TSPs to the thymus and/or generation of ETPs seen in the *Rbp-Jk*-deficient mice (Chapter 3)? Importantly, given the similarity of ETP phenotypes in *Rbp-Jk*- and *Gata3*- deficient mice, whether RBP-Jk is a key regulator of *Gata3* also in ETPs should be investigated.

6

General discussion

Notch is indispensable for the generation of definitive HSCs in the embryo as well as for T cell development in foetal and adult life (Han et al., 2002; Kumano et al., 2003; Radtke et al., 2010; Radtke et al., 1999). Furthermore, *NOTCH1* gain-of-function mutations in humans cooperate with other oncogenic hits to influence T cell acute lymphoblastic leukemia onset and development, and therefore Notch signaling has gained increasing attention as a therapeutic target for tumor treatment (Ferrando, 2009; Palomero and Ferrando, 2009; Radtke et al., 2010). Thus, understanding the role of Notch in lineage commitment and differentiation in the haematopoietic system is pivotal for understanding the mechanisms of disease development and generating the proper tools to treat Notch signaling associated disorders.

6.1 Notch is dispensable for cell lineage commitment in postnatal BM but essential for T cell development in the thymus

Postnatal haematopoiesis occurs mainly in the BM with the exception of T cell development that takes place in the thymus. Physiological properties of BM and thymus may determine HSC and progenitor cell fates. In fact, the intensity of Notch signals differs in the two organs (Felli et al., 1999), where delta-like Notch ligands are expressed at higher levels by the thymic epithelial cells compared to the BM (Felli et al., 1999). In addition, progenitors in the thymus express higher levels of Notch receptors and targets, than BM progenitors (Lai and Kondo, 2007; Mansson et al., 2007; Sambandam et al., 2005). Moreover, the Notch signaling inhibitor LRF represses Notch signals in the BM preventing T cell development in

this organ (Maeda et al., 2007) and allowing B cell lineage commitment to occur. Importantly, the inhibitory effect of LRF on Notch signaling is not observed in the thymus, suggesting that LRF is either repressed in the thymus or its inhibitory effect is overcome by the high levels of Notch ligands expressed by the thymic stromal cells, driving cells to commit towards T cell lineage at the expense of other lineages.

Overall, it seems as if the differential regulation of Notch signaling in the BM and thymus provides the optimal conditions to restrict HSCs or progenitors to differentiate into T cells in the thymus or into other cell lineages in the BM. Accordingly, studies up to the present irrefutably prove Notch signaling to be essential for T cell development in the thymus (Han et al., 2002; Radtke et al., 1999; Sambandam et al., 2005; Tan et al., 2005), however the role of Notch, if any, in the BM remains contentious with contradictory and inconclusive studies reported in the last two decades (Duncan et al., 2005; Maillard et al., 2008; Mercher et al., 2008; Varnum-Finney et al., 2011).

In this thesis work I sought to identify the overall role of Notch in postnatal haematopoiesis. Thus, it is convincingly demonstrated that Notch signaling is dispensable for cell fate specification and development of any myeloid cell lineage in the BM, while its critical role in T cell development in the thymus is further corroborated by the findings I report.

6.1.1 Notch signaling is dispensable for HSC maintenance and function in the BM

In the BM, while different Notch gain-of-function strategies support the idea that Notch signaling can promote expansion of primitive multipotent stem/progenitor cells (Stier et al., 2002; Varnum-Finney et al., 2000), loss-of-function approaches have yielded mixed results as to a potential physiological role of canonical Notch signaling in the regulation of HSCs. Many studies argue against such a role (Han et al., 2002; Maillard et al., 2008; Radtke et al., 1999) whereas other studies support a role for Notch in HSC regulation (Duncan et al., 2005; Varnum-Finney et al., 2011). A dispensable role for Notch signaling in HSCs is further supported here, where population size and function of HSCs are not affected upon disruption of Notch signaling in either steady state or in conditions of stress, such as after myeloablation followed by BM transplantation (see Chapter 2). Importantly, the repopulating capability of BM cells shortly after transplantation was not investigated in this work, however recent studies suggest that fast regeneration of HSCs shortly after BM transfer is NOTCH2 dependent, suggesting that Notch signaling may regulate the HSC expansion that takes place during development and after transplantation (Iscove and Nawa, 1997; Morrison et al., 1995; Pawliuk et al., 1996).

6.1.2 Lineage commitment towards myelo-erythroid lineages occurs independently of Notch signaling

The role of Notch signaling in cell fate determination from HSCs to myeloid lineages in the BM has been widely debated in the field. Gain-of-function studies suggested promoting (Schroeder and Just, 2000; Schroeder et al., 2003) as well as suppressive (Bigas et al., 1998; de Pooter et al., 2006; Milner et al., 1996) effects for Notch in myeloerythropoiesis, but only loss-of-function studies can establish the physiological role of Notch in haematopoiesis. Although initial loss-of-function studies suggested a redundant role of Notch in myeloid differentiation based on a rough readout for myeloid progenitors (Han et al., 2002; Radtke et al., 1999), a recent and more detailed study of the different stages of myeloid progenitors suggested that Mk cell development from HSCs is specified by Notch signaling (Mercher et al., 2008). These results are challenged by my work, by the findings demonstrating that all the myeloid lineages, including Mk cells, are unaffected in population size and function in mice lacking *Rbp-Jk*, and therefore canonical Notch signaling (Chapter 2). Furthermore, Notch is also dispensable for myeloid development after bone marrow transplantation, although in the light of the Varnum-Finney *et al* studies (Varnum-Finney et al., 2011) it would be important to repeat these studies and analyse reconstituted mice sooner after transplantation (Chapter 2).

6.1.3 Initial steps in T cell lineage restriction in the bone marrow occur independently of Notch signaling

The thymus is a non self-renewing organ and therefore needs constant seeding of progenitors coming from the BM (TSPs) to ensure the maintenance of the T cell repertoire in the organism (Goldschneider et al., 1986; Scollay et al., 1986). Given the similarities in cell lineage potential and gene expression signature between LMPPs and ETPs, the former has been suggested as a candidate TSP (Adolfsson et al., 2001; Allman et al., 2003; Mansson et al., 2007; Rothenberg et al., 2008). Phenotypic LMPPs (Jacobsen,SEW and Duarte,S, 2010, unpublished data) are unaffected in the absence of *Rbp-Jk*, thereby failing to show any role of Notch signaling in the generation of candidate TSPs. Nevertheless, other candidate TSPs, such as CLPs, should be investigated and it can also not be ruled out that small but distinct subsets of LMPPs might be affected. The high intensity of Notch signals in the thymus drives TSPs to commit into T cells, to the detriment of other cell lineages (Bell and Bhandoola, 2008; Feyerabend et al., 2009; Han et al., 2002; Pui et al., 1999; Radtke et al., 1999; Wada et al., 2008). Accordingly, I demonstrate here that T cell development in the thymus is impaired in the absence of Notch signaling (Chapter 3).

Overall, studies reported in this thesis show that Notch is dispensable for homeostasis and cell fate determination in postnatal BM and that Notch signaling is likely not to be involved in the establishment of TSPs in the BM but is a determinant for T cell development in the thymus.

6.2 Notch targets are suppressed by RBP-Jk in the absence of Notch signaling

It is clear from studies reported in this thesis as well as studies by others (Lai, 2004; Maillard et al., 2003; Radtke et al., 2010; Radtke et al., 2004) that Notch signaling regulates cell fate determination events in the haematopoietic system, being critical for emergence of HSCs in the embryo (Kumano et al., 2003) as well as T cell development in the adult thymus (Han et al., 2002; Radtke et al., 1999) but dispensable for lineage commitment events in the BM (Maillard et al., 2008). However, studies to date have been largely focused on the loss- and gain-of-function phenotypes observed and little attention has been given to the molecular mechanisms governing these cell fate commitment events. In this work, I investigate for the first time in the haematopoietic system in mammals, how Notch target gene expression levels fluctuate in the presence or absence of Notch signaling, trying to understand how RBP-Jk regulates the activated or repressed status of Notch target genes and if this regulation is dependent upon Notch receptor cleavage or not. It has been previously reported that Notch target genes are up-regulated in the absence of *Su(H)* (RBP-Jk homologue in flies) in *Drosophila* as well as in fibroblasts lacking the *CtBP* Notch signaling co-repressor. Thus, I demonstrate, in mammals *in vivo,* that in the absence of *Rbp-Jk,* transcription of Notch targets is up-regulated in myeloid progenitors in the BM (Chapter 2). Thus, this data indicates that in a “Notch off” state, RBP-Jk actively represses Notch target gene transcription. Although the role of this repressive function of RBP-Jk is not clear as no phenotype is observed, it may be to prevent

ectopic Notch signaling from taking place in the BM, restricting T cell development to the thymus.

It is important to mention that gene derepression of Notch targets is not observed in all cell types. Accordingly, lack of *Lag-1*, the homologue of RBP-Jk in nematodes, does not result in Notch target gene derepression (Ghai and Gaudet, 2008; Neves et al., 2007). Moreover, a gain-of-function phenotype compatible with derepression of Notch targets in the absence of *Rbp-Jk* was not observed in the skin (Demehri et al., 2008) nor in helper T cells (Ong et al., 2008) in mammals. But why does RBP-Jk seem to behave differently dependent on the cell type? One hypothesis is that different Notch target genes have different CSL occupancy periods which would reflect different kinetics of activation. In fact, a recent study showed that in *Drosophila*, binding of Su(H) to the promoter of *E(spl)* is transient and selective (Krejci et al., 2009). Conversely, targets in mammals seem to be almost permanently occupied by the CSL factor (Fryer et al., 2004; Krejci et al., 2009). Of importance, target promoter occupancy by CSL always seems to be enhanced by the presence of NICD (Kopan and Ilagan, 2009). Studies described above suggest that occupancy of Notch target promoters by CSL is a dynamic process which could explain differential Notch target activation. Nevertheless, more studies to test this hypothesis are required. Alternatively, it cannot be excluded that derepression might indicate transcription of Notch target genes by a CSL-independent signaling activation (so called non-canonical Notch signaling).

6.3 RBP-Jk and GATA3: two master regulators for the establishment of ETPs in the thymus

6.3.1 Notch signaling is critical for the establishment of ETPs in the thymus in a NOTCH1-independent manner

Notch signaling is a key regulator of T cell lineage commitment in the thymus (Radtke et al., 2010). However, from which stage in early T cell development Notch starts to be important remains an open question. NOTCH1 has been suggested to be the critical Notch receptor for the generation of ETPs (Sambandam et al., 2005; Tan et al., 2005), which is compatible with ETPs not being found in *Notch1*$^{fl/fl}$ *Mx1-Cre*$^{tg/+}$ mice (Benz et al., 2008). In contrast, using a validated haematopoietic-specific Cre-mediated deletion system, we found that NOTCH1 is not important for the generation of ETPs, but essential for the DN1-DN2 stage transition, with direct repercussions for the subsequent stages of T cell development, when absent (Chapter 3). Contrastingly, no ETPs are generated if canonical Notch signaling is abrogated through deletion of *Rbp-Jk* (chapter 3). All together, these data suggest that canonical Notch signaling is essential for thymus seeding by TSPs and/or establishment of ETPs in the thymus, in a NOTCH1-independent manner. Therefore, the possibility that the lack of an ETP phenotype in *Notch1*-deficient mice may be due to redundancy between Notch receptors cannot be excluded, where the stronger candidate to complement NOTCH1 is NOTCH2 given its important physiological function in the thymus (Benz et al., 2008; Besseyrias et al., 2007), an event previously observed in other developmental pathways (Chen and Zhang, 2001). Alternatively, TSPs in *Rbp-Jk*-

deficient mice may be incapable of reaching and seeding the thymus compromising the downstream generation of ETPs. Thus, further studies are required to understand the phenotypes observed.

Far beyond the scope of the studies reported in this thesis, the identification of the BM TSP that settles in the thymus as well as the role of Notch in its migration from the BM to the thymus would be an important breakthrough in understanding early T cell commitment. Moreover, the role of Notch in thymic ETP stepwise loss of B, myeloid, DC and Nk cell potentials and commitment to the T cell lineage at the cellular and molecular levels remains poorly understood and requires further investigation.

6.3.2 Gata3 is dispensable for HSC maintenance and self-renewal but required for the generation of ETPs in the thymus

GATA3 is also critically important for T cell development (Ho et al., 2009; Pai et al., 2003; Zheng and Flavell, 1997; Zhu et al., 2004), however the role of GATA3 in different stages of early T cell commitment has not been extensively investigated (Hosoya et al., 2009). In this work, I demonstrate that GATA3, in contrast to NOTCH1 but similar to RBP-Jk, is indispensable for the generation of ETPs.

Similar to *Rbp-Jk null* mice, the absence of ETPs in the *Gata3 null* mice may be due to a defect in the migration and/or seeding of the thymus by the TSP coming from the BM. In that regard, the role of Gata3 in the HSC compartment was investigated in detail (Chapter 4), and it is irrefutably shown that GATA3 is

dispensable for HSC self-renewal as well as maintenance in steady-state or in conditions of immune stress (Chapter 4). Further, MPP-containing LSK and LMPP populations were also not affected in size in *Gata3*-deficient mice. All together, these data strongly suggest that GATA3 is not involved in the initial T cell lineage restriction that occurs in the bone marrow or during TSP candidate migration from the BM to the thymus, but does not allow one to exclude the possibility that GATA3 plays a role in thymus seeding events. This requires further investigation.

6.3.3 *Gata3*: target of Rbp-Jk in early T cell commitment?

Notch signaling and GATA3 are critical for T cell development at early stages, including emergence of ETPs in the thymus, which may suggests that RBP-Jk and *Gata3* interact to promote T cell development. Accordingly, RBP-Jk contains DNA binding sites shown to directly bind to the upstream promoter of *Gata3* directly regulating its transcription in T helper cells (Amsen et al., 2007). As reported in these studies, T cell development in *Gata3* and *Rbp-Jk null* mice is seriously compromised and ETPs are hardly detected. Further studies are needed to see if in early stages of T cell development as well, *Gata3* is a direct Notch target gene, where its promoter activation may be directly dependent upon RBP-Jk binding, as observed in Th2 T cells.

6.4 Conclusion

This thesis work brought new insights as to the role of Notch signaling and GATA3 in postnatal haematopoiesis and how these factors act differentially in the BM and thymus, to specifically show:

- Canonical Notch signaling is dispensable for HSC maintenance in steady state and post-transplantation;
- Canonical Notch signaling is not required for steady state and emergency myeloerythropoiesis;
- Notch target genes are kept in a suppressed state by RBP-Jk in bone marrow myeloid progenitor cells;
- Canonical Notch signaling is critical for ETPs in a NOTCH1-independent manner;
- NOTCH1 is indispensable for the DN1 to DN2 transition of T cell development in the thymus;
- GATA3 is redundant for HSC self renewal and maintenance in the BM;
- GATA3 is critical for ETP establishment in the thymus.

7

Materials and Methods

7.1 Mice

7.1.1. Mouse husbandry

Animals used in this thesis work were bred and maintained in pathogen-free animal facilities at the University of Oxford. All procedures and animal handling were approved and licensed by the Home Office and performed under the UK Animals Scientific Procedures Act (1986).

7.1.2. Mouse strains

Rbp-Jk$^{fl/fl}$ mice were obtained from Jonas Frisen (Karolinska Institute, Stockholm, Sweden), after previous agreement with Tasuku Honjo (Kyoto University, Kyoto, Japan) who originally generated the mouse strain (Han et al., 2002). *Notch1*$^{fl/fl}$ mice were provided by Freddy Radtke (Ludwig Institute for Cancer Research, University of Lausanne, Switzerland) (Radtke et al., 1999). *Gata3*$^{fl/fl}$ were generated and imported from Jinfang Zhu´s laboratory (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Maryland, USA) (Zhu et al., 2004). All flox/flox strains mentioned above were backcrossed to a C57BL/6 background and maintained in a heterozygous or homozygous breeding. *Rosa26-YFP* mice were engineered in Frank Costantini´s laboratory (Columbia University Medical Center, USA) (Srinivas et al., 2001) and obtained with agreement from Shoumo Bhattacharya (The Wellcome Trust Centre for Human Genetics, United Kingdom), were backcrossed to a C57BL/6 background and maintained in a heterozygous breeding. Mouse lines above were crossed to

heterozygous or wild type *Mx1-Cre* and/or *Vav-Cre* transgenic mice generated in Klaus Rajewsky´s (Institute for Genetics, University of Cologne, Germany) (Kuhn et al., 1995) and Dimitris Kioussis´s (National Institute for Medical Research, UK) (de Boer et al., 2003) laboratories, respectively. Both Cre lines were maintained on a C57BL/6 background and in a heterozygous or wild-type breeding. Wild-type C57BL/6 and B6-SJL CD45.Ly5.1 mice were bought from Jackson laboratories (Bar Harbor, ME, USA) and were used for backcrossing and transplantation experiments, respectively.

vWF-eGFP BAC transgenic mice, expressing EGFP under control of the endogenous *VWF* promoter, were generated by intracytoplasmic sperm injection and backcrossed to a C57BL/6 background (Sten Eirik W. Jacobsen, and Claus Nerlov, manuscript in preparation).

7.1.3 Genotyping

All mice used in experiments were genotyped by Polymerase Chain Reaction (PCR) analysis using genomic DNA extracted from ear or tail biopsies. Biopsies were digested in 100ul of lysis buffer (LB) (50uL of 50mM Tris pH8, 20uL of 100mM Ethylenediamine tetraacetic acid (EDTA), 2uL of 100mM Sodium Chloride (NaCl), 10uL of 1% Sodium dodecyl sulfate (SDS)) containing proteinase K (pK; Roche) (1 volume pK/25 volumes LB) for 1 hour (h) at 55°C.Then, 28.5ul of 5M NaCl was added and samples spun down for 10min at 14,000 rotations per minute (rpm) at room temperature (RT). Supernatant was added to isopropanol (1:1) to precipitate the DNA. DNA samples were inverted several times and spun

for 15 minutes (min) at 14,000rpm at 4ºC. The pellet was washed with 150ul of 70% ethanol. DNA pellet was air dried and resuspended in 200ul of fresh MilliQ water. One to two microliters of each sample was used directly for the PCR reaction. Genotyping PCR primers used are listed in the table 7.1.

Table 7.1 Mouse genotyping primers

Name	Forward (5´-3´)	Reverse (5´-3´)
Rbp-Jk	ACCAGAATCTGTTTGTTATAT GCATTACTG	ATGTACATTTTGTACTCACAGAGA TGGATG TAATGCACACAAGCATTGTCT GAGTTC
Notch1	CTGACTTAGTAGGGGGAAAAC	AGTGGTCCAGGGTGTGAGTGT
Gata3	CAGTCTCTGGTATTGATCTG CTTCTT	GTGCAGCAGAGCAGGAAAC TCTCAC
vWF	CCTCTCTGGACGGTGAGAAC	AAGTCGTGCTGCTTCATGTG
GFP	AGCAAGGGCGAGGAGCTGTT	GTAGGTCAGGGTGGTCACGA
Mx1-Cre	CGTTTTCTGAGCATACCTGGA	ATT CTC CCA CCG TCA GTA CG
Vav-Cre	AGATGCCAGGACATCAGG AACCTG	ATCAGCCACACCAGACACA GAGATC
Rosa26 WT	GGAGCGGGAGAAATGGATATG	AAAGTCGCTCTGAGTTGTTAT
Rosa26 YFP	CGTAAACGGCCACAAGTTCAG	GAACTCCAGCAGGACCATGTG

7.1.3.1 Polymerase chain reaction (PCR)

PCR reactions were specifically optimized for each genotype. Typically, PCR reactions were prepared in a total reaction volume of 20-25uL, containing forward and reverse primers (0,5% of final volume, at 10 or 100uM; Invitrogen), Taq polymerase enzyme (Invitrogen), 10x buffer supplied with the Taq polymerase, 50mM of Magnesium Chloride ($MgCl_2$), deoxyribonucleotide triphosphate (dNTP) nucleotides, distilled water and at most 2ul of template DNA. The PCR reaction was carried out on a thermal cycler (BioRad, Tetrad II 96-Well Alpha Unit) with an initial heat activation step at 94-95ºC for 2-10 minutes followed by a DNA denaturation step (94-95ºC for 30 seconds to 1 minute), an annealing step (54-65ºC for 30-90 seconds), elongation (25-45 cycles at 72ºC for 30-120 seconds) and a final extension at 72ºC for 5-20 minutes.

7.1.3.2 Agarose gel electrophoresis

PCR products were separated by electrophoresis on 2% agarose gels, prepared with agarose powder (Invitrogen) dissolved in 1x tris-Acetate EDTA (TAE) buffer prepared in house. Sybr green (5ul per 50ml; Invitrogen) or ethidium bromide (2 drops per 100 microliters of agarose gel; Dutscher Scientific) was added and products were visualized by illumination under ultra-violet (UV) light.

7.2 *In vivo* assays and procedures

All *in vivo* procedures were performed under sterile conditions and in a laminar flow hood.

7.2.1 Poly(I:C) treatment

Flox/flox mice crossed to *Mx1-Cre* mice were intraperitoneally injected with poly(I:C) (GE Healthcare) every second day, for 6 days, at a dose of 200ug per injection to induce Cre-mediated recombination.

7.2.2 Mouse tail vein blood collection

Mice were warmed up in a heat box and placed in a sterilized mouse restrainer. A gentle cut across the lateral vein was done using a scalpel and blood was collected into an EDTA-coated tube (Sarstedt).

7.2.3 Intravenous transplantation

The CD45 isoforms 1 and 2 are used to distinguish donor versus recipient cells. Typically, recipient (CD45.1 or CD45.1/2) mice were lethally irradiated with 900 centiGray (cGy) and competitively transplanted with 0.5-2 millions of test BM cells (CD45.2) along with 0.5-2 millions of competitor (CD45.1 or CD45.1/2) BM cells (1:1), by injecting them in the tail vein. For secondary transplantations, half-femur equivalent BM cells from primary recipients were transplanted into lethally irradiated secondary wild type recipients. Positively reconstituted mice had a

minimum of 0.1% test cell contribution relative to total nucleated cells, and a minimum of 0.02% to each of the myeloid, B and T cell lineages.

7.3 Flow cytometric analysis and cell sorting

7.3.1 Preparation of single cell suspensions

For BM studies, tibiae and femurs were harvested from individual mice into phosphate buffered saline (PBS; Dulbecco) supplemented with 5% fetal calf serum (FCS; ThermoFisher). Cleaned bones were crushed in a mortar. Cells were disaggregated by gentle pipetting several times with PBS with 5% FCS. The cell suspension was filtered through a cell strainer to remove cell clumps. For studies with thymocytes, thymi were harvested, cleaned of fat tissue and blood and disrupted by passing the tissue through a 70μm mesh filter (Becton, Dickinson and Company, BD). Cells were washed and resuspended in PBS with 5% FCS. Viable BM cells and thymocytes (2 replicates) were counted using Trypan blue (Sigma) complemented with Zapaglobin II (Beckman Coulter) or by using a Sysmex hemocytometer (KX-21N, Sysmex). All steps were performed on ice.

7.3.2 White blood cells isolation from mouse peripheral blood

Two per cent Dextran (Sigma) solution in PBS was added to each blood sample at a 1:1 proportion and gently mixed by pipetting up and down. Cells were incubated for 20-25 min at 37ºC for red blood cell sedimentation, upon which the upper phase was transferred into a clean collection tube, washed and spun down

(3200rpm, 4min, RT). Supernatant was discarded and pellet dissolved in 0.2 ml of Ammonium Chloride (NH_4Cl) for 1 minute to lyse the erythrocytes. Cells were finally washed with PBS supplemented with 1% FCS.

7.3.3 Platelets isolation from mouse peripheral blood

Each blood sample was spun down at 1000 rpm for 10 min at 4ºC. Platelets-containing upper phase was collected and stained for FACS analysis.

7.3.4 Quantification of circulating blood platelets

Peripheral blood was collected from the tail vein of individual mice into EDTA-coated tubes. Platelet counts per milliliter of blood were quantified on a Sysmex® automated hematology analyzer (KX-21N, Sysmex).

7.3.4 CD117/c-KIT$^+$ cell enrichment

Bone marrow and thymic CD117/c-KIT$^+$ stem and progenitor cells were enriched prior to sorting by magnetically labeling them with anti-CD117 MicroBeads (MACS) following manufacturer instruction with minor modifications (Miltenyi Biotech). Single cell suspension was spun down (500g, 10 min, 4ºC) and resuspended in a buffer containing PBS with 5% FCS at a concentration of 100 million cells per 100ul of total volume. Two and a half microliters of CD117 MicroBeads per 100 million cells were next added and cells were incubated for 20 minutes on ice, in dark and on a rotor, to avoid cell sedimentation. Cells were

washed, spun down (500g, 10 min, 4ºC) and resuspended in 3ml of buffer. A MACS LS column provided by the manufacturer (Miltenyi Biotech) was placed in the magnetic field of a MACS Separator. The column was equilibrated with buffer prior use. Cells magnetically labeled with anti-CD117 beads were filtered and loaded onto the column (the magnetically labeled $CD117^+$ cells are retained within the column and the unlabeled $CD117^-$ cells run through the column). The column was washed with 3 ml of buffer for 3 times and the flow-through discarded (columns were not loaded with more than 1×10^9 cells). The column was next removed from the separator, placed on a suitable collection tube and 3-5ml of buffer was added. The magnetically labeled cells were flushed out by pushing the plunger provided by the manufacturer into the column. Cells were finally counted with an expected $CD117^+$ cells recovery of 4-6% for the BM, and 0.5-1% for the thymus.

7.3.5 Antibody staining

For all staining steps, cells were incubated with specific antibodies in PBS supplemented with 5% FCS for at least 15 minutes on ice and in dark followed by a washing step with the same buffer.

Specifically, for FACS analysis of myeloid progenitors (PreGM, GMP, PreMegE, MkP, Pre-CFU-E, CFU-E, ProEry; chapter 2), cells were stained with lineage marker purified rat anti-CD4 (H129.19; Becton Dickinson (BD)), anti-CD8 (F3-6.7; BD), anti-Mac1 (M1/70; eBioscience), anti-Gr1 (RB6-8C5; BD), and anti-B220 (RA3-6B2; BD) and subsequently stained with goat anti-rat-QD605 (MP,

Invitrogen). Cells were further stained with anti-CD16/32-PE (93; eBioscience), anti-Ter119-PECy5.5 (TER119; eBioscience), anti-CD41-PECy7 (MWReg30; eBioscience), anti-CD150-APC (TC15-12F12.2; BioLegend), anti-c-Kit-APC-eF780 (2B8; eBioscience), anti-Sca1-Pacific Blue (E13-161.7; BioLegend) and anti-CD105-biotin (MJ71/18; BioLegend). For analysis of HSCs (chapters 2, 4 and 5), cells were stained with the lineage markers above and further stained with anti-CD150-APC (TC15-12F12.2; BioLegend), anti-c-Kit-APC-eF780 (2B8; eBioscience), anti-Sca1-FITC (E13-161.7; BioLegend) and anti-FLT3-PE (A2F10; BD or BioLegend). Alternatively, cells were stained with anti-CD150-PECy7, anti-c-Kit-APC-eF780, anti-Sca1-FITC and anti-CD48-APC (Biolegend). Biotinylated antibodies were visualized with Streptavidin PE Texas Red (BD). Finally, for analysis of ETPs, c-KIT^{+} DN2s and DN3s (chapters 3 and 5), cells were stained with APC-conjugated lineage antibodies anti-CD3ε (145-2C11, eBioscience), anti-TCRγ (GL3, eBioscience), anti-Gr-1, anti-NK1.1, anti-TCRβ (H57-597, eBioscience), anti-CD11c (N418, eBioscience) and subsequently stained with anti-CD44-FITC (IM7, BD), CD4-AF700 (RM4-5, eBioscience), c-KIT-APC-eF780, anti-FLT3-PE, anti-CD25-PerCPCy5.5 (PC61, BD), anti-B220-PE-Texas Red (BD) and CD8α-PECy7 (53-6.7, eBioscience).

For analysis of HSCs in CD45.1 or *vWF-eGFP* CD45.1/2 transplanted mice (chapter 2), cells were lineage stained with purified rat anti-CD5 (53-7.3; eBioscience), anti-CD8a, anti-B220, anti-Ter119 and anti-Gr1, and further stained with goat-anti-rat-tricolor (Invitrogen). Cells were then stained with anti-CD45.1-PE (A20; eBiosciences), anti-CD45.2-AF700 (104; eBioscience), anti-FLT3-Biotin, anti-c-Kit-APC-eF780, anti-CD150-APC and anti-Sca-1-PB. Biotinylated antibodies

were visualized with Streptavidin PE Texas Red. Donor-derived myeloid progenitor BM cells were lineage stained (CD4, CD8, Mac-1, Gr1 and B220) and then incubated with goat-anti-rat-QD605. Subsequently, the following antibodies were used: anti-CD45.1-PECy5, anti-CD45.2-AF700, anti-CD16/CD32-PE, anti-Ter119-PECy5.5, anti-CD41-PECy7, anti-CD150-APC, anti-c-Kit-APC-eF780, anti-Sca-1-PB and anti-CD105-biotin. Biotinylated antibodies were visualized with Streptavidin PE Texas Red.

Platelets in the blood of reconstituted mice (chapter 2) were harvested in EDTA-coated tubes, isolated and stained with anti-CD41-PECy7 and anti-CD150-APC.

FACS analysis of peripheral blood from transplanted WT recipients (chapter 4) was done by staining cells with rat-anti-CD45.1-PE, anti-CD45.2-AF700, anti-Gr1-Pacific Orange (RB6-8C5; Caltag), anti-CD19-PECy7 (1D3; eBioscience), anti-Mac1-APC (M1/70; Biolegend), anti-CD4-APCeF780 (RM4-5; eBioscience), anti-CD8-APC-eF780 (53-6.7; eBioscience), and anti-NK1.1-Pacific Blue (PK136; Biolegend).

For all analysis, dead cells were excluded with 7-amino-actinomycin D (7-AAD, Sigma) or 4,6-*diamino*-2-phenylindole (DAPI; Invitrogen).

FACS analyses were done on a LSR II analyzer (BD Biosciences) and sorts on a FACSAria IIu Special Order Research Products (BD Biosciences). Sorted cells were consistently 97-99% pure as determined by reanalysis.

7.5 Mouse colony assays

7.5.1 In vitro mouse BFU-E and CFU-GM potential assay

For evaluation of E and GM clonal potential, 50,000 unfractionated BM cells (2 replicates per sample) were seeded in 1 mL of complete methylcellulose (GF M3434; StemCell Technologies) supplemented with mouse stem cell factor (mSCF; 50ng/mL), mouse interleukin-3 (mIL-3; 10ng/mL), human IL-6 (hIL-6; 10ng/mL) and human erythropoietin (hEPO; 3U/mL). Cells were cultured for 7 days in an incubator with humidified atmosphere, >95% humidity, 5% CO2 at 37 °C, at which time erythroid potential was evaluated using 2,7-diaminofluorene (DAF; Sigma-Aldrich) staining (DAF stock solution (10mg/ml): 100 mg DAF were dissolved in 10ml 90% glacial acetic acid/d$H_2$0). DAF staining solution was prepared by adding half milliliter of DAF stock solution to 0.1mL of 30% of Hydrogen peroxide (H_2O_2) and 10mL of 200mM of Tris Hydrochloric acid (HCl). DAF-positive cells were identified as cells with intracellular blue granules. BM-derived pure GM clones were generated in methylcellulose (M3134; SCT) and supplemented in house with the myeloid cytokines 5ng/mL mouse granulocyte-monocyte colony stimulating factor (mGM-CSF; Amgen Corp), 10ng/mL human fms-like tyrosine kinase 3 ligand (hFL; Immunex), 10ng/mL human granulocyte colony stimulating factor (hG-CSF; Amgen Corp) and 2ng/mL mIL-3, and used as a negative control to confirm the specificity of the DAF staining. Colonies were visualized and counted using a common inverted microscope (CKx41, Olympus).

7.5.2 *In vitro* mouse CFU-Mk potential assay

For assessment of Mk potential, BM cells (100,000 cells/slide) were mixed into Megacult (SCT) supplemented with Collagen (SCT), and 50 ng/mL human thrombopoietin (hTHPO; PeptroTech), hIL-11 (Genetics), 10 ng/mL mIL-3 and 20 ng/mL hIL-6 (PeproTech), and cultured in chamber slides (SCT) at 37°C in an incubator with an atmosphere of 5% CO_2 and >95% humidity for 7 days. Slides were then dehydrated and fixed with cold acetone and stained with staining solution of acetylthiocholiniodide (Sigma) dissolved in 0.1 M sodium phosphate buffer added to 0.1 M sodium citrate, 30 mM copper sulphate and 5 mM potassium ferricyanide solution for 3-6 hours and fixed with 95% ethanol for 10 minutes. Colonies were counterstained with Harris' hematoxylin (Sigma) for 30 seconds. Colonies were counted using a regular inverted microscope (CKx41, Olympus). CFU-Mk colonies were identified as brown colored colonies and ranged in size from 3 to approximately 50 megakaryocytes per colony.

7.6 Gene expression analysis

7.6.1 Quantitative gene expression

Multiplex real time PCR analysis was performed using BioMark 48.48 Dynamic Array platform (Fluidigm) and TaqMan Gene Expression Assays (Applied Biosystems). For all analysis, 100 bone marrow HSCs, myeloid progenitors (PreGM, GMP, PreMegE, MkP, Pre-CFU-E, CFU-E) or thymic ETP, c-KIT$^+$DN2 and DN3 progenitor cells were sorted directly into 0.2mL PCR tubes containing

2.5uL gene specific 0.2x TaqMan gene expression assay mix (Applied Biosystems), 5uL CellsDirect 2x reaction mix (Invitrogen), 1.2uL CellsDirect RT/Taq mix, 1.2uL TE buffer and 0.1uL SUPERase 12-In RNase inhibitor (Ambion), in a total volume of 10uL. Reverse transcription (RT) and specific target amplification (STA) were then carried out on a thermal cycler using the following conditions: RT at 50ºC for 15 minutes; inactivation of RTase/activation of Taq enzyme at 95ºC for 2 minutes; STA with 22 cycles of 95ºC for 15 seconds and 60ºC for 4 minutes. Pre-amplified complementary DNA (cDNA) was diluted with TE buffer (1:5; Sigma). Quantitative gene expression analysis was performed using the BioMark™ 48·48 Dynamic Array (Fluidigm) with the following PCR cycling condition: 95°C for 10 minutes; 40 cycles of 95°C for 15s and 60°C for 60s. Data were analyzed using BioMark™ Real-Time PCR Analysis Software v2.0 (Fluidigm) and the ΔCt method applied (Livak and Schmittgen, 2001). Results were normalized to *Hprt* expression and expressed as mean expression levels relative to *Hprt*. In general, gene expression levels below 0.001 (relative to *Hprt*) were only intermittently detected and therefore 0.001 was defined as the cut-off value. The TaqMan® Gene Expression Assays used in this analysis are listed in table 7.2 below.

Table 7.2 TaqMan® Gene Expression Assays

Gene	Applied Biosystems Assay ID
Cebpα	Mm00514283_s1

Cd25/ Il2rα	Mm00434261_m1
EpoR	Mm00438760_m1
Etv6/Tel-1	Mm00468390_m1
Flt3 receptor	Mm00439016_m1
Fog1/zfpm1	Mm00494336_m1
Gata1	Mm00484678_m1
Gata2	Mm00492300_m1
Gata3	Mm00484683_m1
Gata3 (deletion)	Mm01337569_m1
G-csf receptor/csf3r	Mm00432735_m1
Gm-csf receptor α/csf2rα	Mm00438331_g1
Hes1	Mm01342805_m1
Hes5	Mm00439311_g1
Hprt1	Mm00446968_m1
Klf1	Mm00516096_m1
Lrf/zbtb7α	Mm00657132_m1
Mint/spen	Mm00465639_m1
Mpl	Mm00440310_m1
Mpo	Mm00447886_m1
Nfe2	Mm00801891_m1
Notch1 receptor	Mm00435245_m1
Notch1 receptor (deletion)	Mm00627185_m1
Notch2 receptor	Mm00803077_m1

Nrarp	Mm00482529_s1
Rbp-Jk (deletion)	Mm01217627_g1
Runx1	Mm01213405_m1
Runx3	Mm00490666_m1
Scl/Tal-1	Mm00441665_m1
Vwf	Mm00550376_m1

7.6.2 Global Gene expression

Global gene expression analysis was performed on HSCs, LMPPs and ETPs. For each population, three biological replicates – individually sorted samples from different mice – were prepared. Cells were sorted directly into Trizol (Invitrogen) and the RNA extraction carried out as per the manufacturer's instructions, with the exception that linear polyacrylamide (GenElute-LPA, Sigma-Aldrich) was used as the carrier instead of glycogen. Samples were then amplified using the Nugen kit WTOvation Pico RNA Amplifications System followed by the WT Ovation cDNA Biotin Module V2 for cDNA labeling and fragmentation and finally hybridized to Affymetrix Mouse Genome 430 2.0 Arrays using standard protocols (Affymetrix) at the Stanford Protein and Nucleic Acid facility. Data were normalized using the Robust Multi-array Averages (RMA) method in the *affy* package.

7.7 Statistical analysis

The statistical significance of differences between samples was determined using the 2-tailed Mann-Whitney test.

For quantitative gene expression analysis, mean values of 2-3 replicates per individual mouse per cell population were determined. The mean values of each mouse were then used to calculate the mean (SEM) variation between individual mice and statistical significance.

8

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