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ELUCIDATING REGULATORY NETWORKS PROMOTING B-CELL DEVELOPMENT

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Elucidating regulatory networks promoting B-cell development

Abstract

B-cells are an essential part of our adaptive immune system. A network of transcription factors together with external signals facilitate the gradual developmental progression from progenitor cells towards the B-cell fate. Several key factors participating in this network have been identified. Among these are the transcription factors E2A, HEB, EBF1 and FOXO1 as well as the IL7 signaling cascade.

The objective of this thesis has been to increase our understanding of the transcriptional network orchestrating B-lineage specification and commitment. Based on the combined expression of surface markers and transgenic reporter genes, we have identified three hierarchically related and functionally distinct subpopulations within the common lymphoid progenitor (CLP) compartment. Using this knowledge, we have re-evaluated previously characterized knock-out mouse models in order to obtain a higher resolution analysis of critical events in early B-cell commitment. Based on these studies, we propose a transcriptional hierarchy where the transcription factors E2A and HEB initiate the B-cell specification program in the LY6D- CLPs through up regulation of FOXO1. During the transition to LY6D expressing CLPs, E2A and FOXO1 induce EBF1. Subsequently, FOXO1 and EBF1 generate a feed-forward loop, leading to activation of PAX5, B-cell commitment and the progression to the CD19+ pro-B cell stage.

Key words: Hematopoiesis, B-cell development, common lymphoid progenitor

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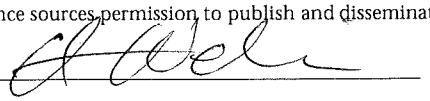
Signature  Date 2012-04-27

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Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity.
Blood. 2010 Apr 1;115(13):2601-9

Paper II

Welinder E, Mansson R, Mercer EM, Bryder D, Sigvardsson M, Murre C
E2A and HEB Act in Concert to Induce the Expression of FOXO1 in the Common Lymphoid Progenitor
Proc Natl Acad Sci U S A. 2011 Oct 18;108:17402-7

Paper III

Robert Mansson*, **Eva Welinder***, Josefine Ahsberg, Yin Lin, Christopher Brenner, Christopher K. Glass, Joseph Lucas, Mikael Sigvardsson and Cornelis Murre
The Transcriptional Regulators, FOXO1 and EBF1, Establish a Feed-Forward Loop to Orchestrate the B cell Fate
Submitted manuscript
*These authors contributed equally

Listed below are published work performed during my doctorate that are not included in this thesis.

Lin YC, Jhunjhunwala S, Benner C, Heinz S, **Welinder E**, Mansson R, Sigvardsson M, Hagman J, Espinoza CA, Dutkowski J, Ideker T, Glass CK, Murre C.
A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate.
Nat Immunol. 2010 Jul;11(7):635-43.

Welinder E, Murre C.
Ldb1, a new guardian of hematopoietic stem cell maintenance.
Nat Immunol. 2011 Feb;12(2):113-4.

Tsapogas P, Zandi S, Åhsberg J, Zetterblad J, **Welinder E**, Jönsson JI, Mansson R, Qian H, and Sigvardsson M.
IL-7 mediates Ebf-1 dependent lineage restriction in early lymphoid progenitors.
Blood. 2011 Aug 4;118(5):1283-9

Welinder E, Ahsberg J, Sigvardsson M.
B-lymphocyte commitment: identifying the point of no return.
Semin Immunol. 2011 Oct;23(5):335-40.

ABBREVATIONS & SYNONYMS

B29	CD79b molecule, immunoglobulin-associated beta (CD79b)
Bcl2	B-cell leukemia/lymphoma 2
BCR	B-cell receptor
Blnk	B-cell linker
BM	Bone marrow
Bmi1	Bmi1 polycomb ring finger oncogene
BSAP	B-cell specific activator protein
ChIPseq	Chromatin immunoprecipitation coupled to deep sequencing
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRC	Chromatin remodeling complex
Dnmt1	DNA methyltransferase 1 (cytosine-5)
EBF1	Early B-cell factor 1
ESC	Embryonic stem cell
ELP	Early lymphoid progenitor
ETP	Early thymic progenitor
FLT3	FMS-like tyrosine kinase 3
FOXO1	Forkhead box O1
GMP	Granulocyte macrophage progenitor
Gr1	Lymphocyte antigen 6 complex, locus G (Ly6g)
H3K4me1	Monomethylation of histone H3 at lysine 4
H3K4me3	Trimethylation of histone H3 at lysine 4
HSC	Hematopoietic stem cell
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
Ikaros	IKAROS family zinc finger 1 (Ikzf1)
IL7r	Interleukin 7 receptor
Kit	Kit oncogene
KO	Knock-out
λ5	Immunoglobulin lambda-like polypeptide 1 (Igll1)
Lin$^-$	Lineage negative (e.g. lineage marker negative)
LMPP	Lymphoid primed multipotent progenitor
LRF	Leukemia related factor (Zbtb7a)
LSK	LIN$^-$SCA1$^+$KIT$^+$
LSK$^-$	LIN$^-$SCA1$^+$KIT$^-$
LY6C	Lymphocyte antigen 6 complex, locus C
LY6D	Lymphocyte antigen 6 complex, locus D
MAC1	Integrin α M (CD11B/CD18)
Mb1	CD79a molecule, immunoglobulin-associated alpha (CD79a)
MCSFR	Colony stimulating factor 1 receptor (Csf1r)
MkE	Megakaryocyte-erythrocyte
MPP	Multipotent progenitor

NOTCH	Notch gene homolog
NuRD	Nucelosome remodeling and deacetylase
PAX5	Paired box gene 5
PI(3)K	Phosphatidylinositol 3-kinase
PRC	Polycomb repressor complex
PU.1	SFFV proviral integration 1 (Sfpi1)
RAG1/2	Recombination activating gene 1/2
SCA1	Stem cell antigen-1 (Ly6a)
SWI/SNF	Switch/Sucrose NonFermentable
Tdt	Deoxynucleotidyltransferase, terminal (Dntt)
TER119	Lymphocyte antigen 76 (Ly76)
TSS	Transcriptional start site
Vpreb1/2/3	Pre-B lymphocyte gene 1/2/3
Znf521	Zink finger protein 521

Official gene symbols are indicated in parenthesis when alternative nomenclature has been used in the text. Molecules usually referred to by their CD number have not been included in the list of abbreviations. Genes and mRNA products are referred to in the text by the name written in italics while corresponding protein products are written in capital letters.

BACKGROUND

INTRODUCTION

The major task of B-cells is to produce antibodies against foreign antigens so that they can be recognized and cleared by other cells of the immune system. Extensive research efforts during the last decades have rendered the B-cell differentiation pathway among the best understood developmental processes to date. However, we still lack some fundamental knowledge about this pathway preventing us from fully understanding underlying causes of pathological conditions and from gaining the ability to treat immunodeficiency and B-cell related malignancies. Biological developmental processes are highly complex and tightly regulated. Among elements guiding cells during these processes are signals from neighboring cells and cytokines, regulation by transcription factors and factors influencing epigenetic patterning and chromatin structure.

The focus of this thesis has been to improve our understanding of the regulatory network generated of transcription factors that guides a hematopoietic progenitor cell in the bone marrow towards a B-lymphoid cell fate. The work adds to previous studies by refining the scheme for developmental progression towards the B-cell lineage just prior to B-cell commitment. These novel intermediate developmental stages have, in the context of transcription factors known to influence B-lymphocyte development, been studied in detail to fine tune our understanding of early B-cell differentiation.

HEMATOPOIESIS & THE HEMATOPOIETIC TREE

Hematopoiesis is the fundamental biological process during which new blood cells are being formed. These blood cells mature to participate in highly specialized and essential functions, such as oxygen transportation (erythrocytes) wound healing through blood clotting (platelets) and immune defense (lymphocytes, granulocytes and macrophages) (Figure 1). The majority of the mature blood cells are short-lived (hours to weeks), thus there is a great need to constantly generate new hematopoietic cells. During steady-state blood homeostasis, about 10^{11}-10^{12} new blood cells are generated in an adult human each day (Ogawa, 1993).

Pioneering work performed during the mid 20^{th} century indicated that the replacement of blood cells was based on differentiation of hematopoietic stem cells (HSCs) in the bone marrow (BM) of mice. A series of experiments was performed by James Till, Ernest McCulloch and colleagues where bone marrow cells were transferred to irradiated host mice and extensively assayed. These experiments indicated the presence of cells with potential to reconstitute the entire spectrum of hematopoietic cells allowing for the establishment of the HSC concept (Becker *et al*., 1963, Siminovitch *et*

al., 1963). The proof-of-existence of a common progenitor for all blood cells was further developed during the 1980s where retroviral labeling of cells, demonstrated that the same integration site could be identified in multiple mature lineages (Dick *et al*., 1985; Keller *et al*., 1985; Lemischka *et al*., 1986). The formal proof for the existence of a true HSC was obtained when single cell transplantations confirmed a common ancestry for the entire hematopoietic system (Osawa *et al*., 1996). The HSC activity was early shown to reside within a population of cells displaying little or no surface expression of lineage markers found on mature cells and high levels of SCA1 and KIT (LSK) cells (Spangrude *et al*., 1988, Ikuta and Weissman, 1992). Extensive characterization of the LSK-population has demonstrated it to be highly heterogeneous and several additional surface markers including CD34, CD150 and FLT3 has been added to the scheme to further enrich for or exclude stem cell activity (Osawa *et al*., 1996, Christensen and Weissman, 2001, Adolfsson *et al*., 2001, Kiel *et al*., 2005). Recently, "true" HSCs have been suggested to be heterogeneous, containing epigenetically primed subsets of myeloid or lymphoid biased cells (reviewed by Schroeder, 2010). In order to maintain homeostasis the HSCs are constantly in a balance between quiescence, self-renewal and differentiation.

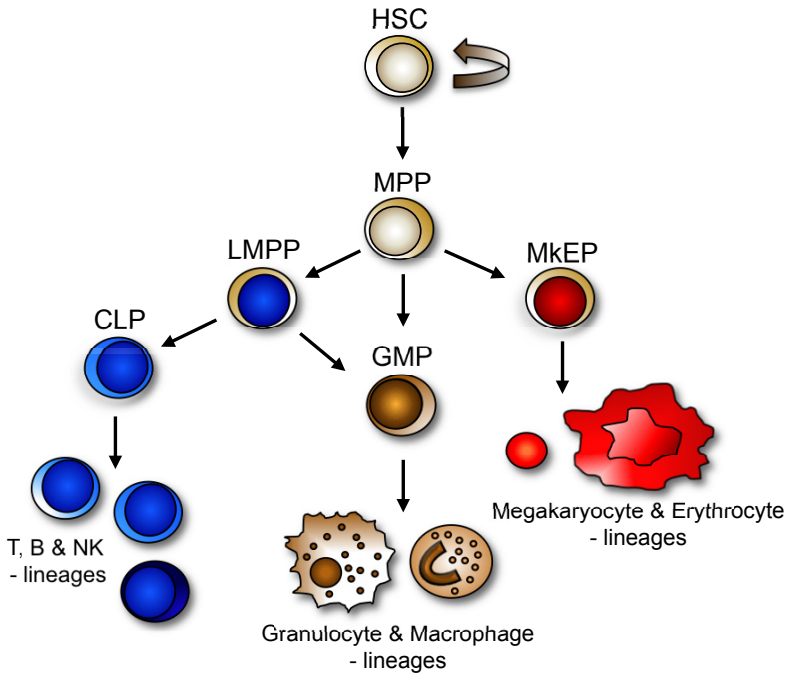

FIGURE 1. Schematic representation of the hematopoietic tree. HSC: hematopoietic stem cell, MPP: multipotent progenitor, MkEP: megakaryocyte and erythrocyte progenitors, GMP: granulocyte-macrophage progenitor, LMPP: lymphoid-primed multipotent progenitor, CLP: common lymphoid progenitor; T: T-cell, B: B-cell, NK: natural killer cell.

The most immature differentiated progeny are referred to as multipotent progenitors (MPPs) and they have lost the ability to self-renew but remain potent to short term reconstitution of the entire hematopoietic system (Morrison *et al*., 1997). As the MPPs divide and differentiate further the daughter cells gradually lose the potential to generate progeny of multiple lineages and finally commit to a certain cell fate. The classical model of hematopoiesis proposed a mechanism where the MPPs give rise two lineage restricted populations named common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) (Kondo *et al*., 1997a, Akashi *et al*., 2000). This suggests a strict and early separation of the myeloid and lymphoid differentiation pathways (summarized by Reya *et al*., 2001).

The routes of the lineage restriction events of the MPPs is however a topic under constant development and debate. Throughout the years several models have been proposed and the classical model was most recently challenged by the identification of a lymphoid primed multipotent progenitor (see below) showing combined lymphoid and myeloid potential (reviewed by Kawamoto and Katsura, 2009 and Ceredig *et al*., 2009). In addition, the CMP population has been shown to be highly heterogeneous, appearing to consist of a mixture of cells representing various developmental stages of cells from several cell lineages (Pronk *et al*., 2007).

BONE MARROW B-CELL DEVELOPMENT

Lymphoid primed multipotent progenitors

The first cells appearing *en route* towards a lymphoid cell fate can be found within a fraction of LSK cells expressing high surface levels of the fms-like tyrosine kinase 3 (FLT3) (Adolfsson *et al*., 2005, Mansson *et al*., 2007). The upregulation of FLT3 occurs in MPPs and coincides with loss of self-renewing properties and $FLT3^{high}$ cells have lost the ability to properly reconstitute the megakaryocyte and erythrocyte (MkE) lineages (Christensen and Weissman, 2001, Adolfsson *et al*., 2001, Adolfsson *et al*., 2005, Lai and Kondo, 2006). These $FLT3^{high}$ cells were referred to as lymphoid primed multipotent progenitors (LMPPs) and exhibited lineage priming with low transcriptional expression of lymphoid-associated genes such as *Tdt*, *Rag1* and *Rag2*. While these cells expressed common lymphoid genes no expression of B or T cell specific genes could be detected (Adolfsson *et al*., 2005, Mansson *et al*., 2007). Even though the exact role of FLT3 signaling in lineage restriction events remains unclear the importance of proper FLT3 signaling for development of lymphoid cells is stressed by the notion that lymphoid progenitors are reduced in mice lacking FLT3 signaling (Mackarehtschian *et al*., 1995, McKenna *et al*., 2000, Sitnicka *et al*., 2002).

The link between transcription of lymphoid genes and lymphoid lineage restriction was also supported by analysis of the LSK compartment in a RAG1-GFP reporter mouse. This allowed for the isolation of a small GFP-expressing population of cells composing about 3-5% of the total LSKs (Igarashi *et al*., 2002). These cells are rarer than the LMPPs that represents about 25% of the LSK-compartment. However, these cells showed lymphoid lineage priming and had limited myeloid-erythroid potential but could readily give rise to B, T and NK cells. These cells where denoted early lymphoid progenitors (ELPs) and most likely represents a more mature lymphoid-differentiated LMPP population. Recently, a cell population with LMPP-like features was identified in human hematopoiesis as well suggesting that the basic structures for the hematopoietic tree is similar in mouse and humans (Doulatov *et al*., 2010).

Common lymphoid progenitors

Subsequent differentiation of LMPPs towards a lymphoid cell fate likely results in the development of the common lymphoid progenitor (CLP) population (Adolfsson *et al*., 2001). The CLPs were originally identified as lineage negative, $SCA1^{lo}$, KIT^{lo}, Interleukin-7 receptor α (IL7r) expressing cells with the ability to generate B, T and NK cells with limited myeloid potential (Kondo *et al*., 1997). This CLP population was

further refined using a combination of the surface receptors IL7r and FLT3 (Karsunky *et al*., 2008). Both FLT3 and IL7r signaling are independently necessary for the normal generation of B-cell precursors. Further, the combined loss of both signaling pathways results in a more severe phenotype as compared to the loss of either of these alone, indicating that these cytokines may act in a collaborative manner in the generation of lymphoid precursors (Veiby *et al*., 1996, Sitnicka *et al*., 2003, Åhsberg *et al*., 2010).

In order to explore the B-lineage pathway from the CLP, a reporter strain carrying human CD25 under the regulatory regions of the surrogate light chain gene immunoglobulin lambda-like 1 (Igll1, also known as λ5) locus has been utilized. This reporter was demonstrated to specifically mark early B-lineage progenitors (Martensson *et al*., 1997). However, within the CLP compartment, a small reporter positive population, lacking both the surface markers CD19 and B220, displayed *in vitro* commitment to the B-cell lineage (Mansson *et al*., 2008). The λ5 reporter was further crossed into the RAG1-GFP reporter strain previously used to identify the ELP-population (Igarashi *et al*., 2002). Combining the reporters, the CLP compartment could be divided into three hierarchically related and functionally distinct populations. Cells lacking expression of both reporters possessed T, NK as well as B-lineage potential, while expression of the RAG1 reporter was associated with a loss of NK-cell potential. The λ5 reporter gene was only expressed in a subpopulation of the RAG1 expressing cells and represented cells largely restricted to B-lineage development. The lineage potentials of these populations suggests that lymphoid lineage restriction involves a sequential loss of first NK- and then T-lineage potential coupled with progressively faster kinetics for the generation of $CD19^+$ B-cells (Paper I). Transcriptional profiling of these three populations identified the surface marker LY6D to be enriched in the RAG1 expressing CLPs and FACS analysis verified that surface expression of LY6D marks cells with limited NK lineage potential (Paper I). Around the same time Inlay and colleagues independently reported that surface expression of LY6D could divide the CLP compartment into two functionally distinct populations, where the LY6D positive cells represented cells preferentially generating B-lineage cells after transplantation (Inlay *et al*., 2009). Further, using a fate-mapping model where cells with a history of RAG1 expression where permanently marked through the expression of Cre under the regulatory elements of RAG1, the CLP compartment, as expected, showed heterogeneous labeling (Welner *et al*., 2009). Marked CLPs were less likely to produce natural killer (NK) and dendritic cells (DC) than unmarked CLPs in *in vitro* cell cultures and labeled CLPs developed faster into $CD19^+$ cells (Welner *et al*., 2009). All-in-all this suggests that the CLP population is highly heterogeneous, containing several related developmental stages as well as cells already committed to the B-cell lineage.

The CLPs *in vivo* contribution to non B-lymphoid cells has been questioned. It is not well understood which progenitor population(s) from the bone marrow that seed the thymus. It is, however, clear that CLPs can readily generate T-cells in stromal cell co-

culture in the presence of Notch-signaling and when transplanted into irradiated hosts (Schmitt and Zuniga-Pflucker, 2002, Serwold *et al*., 2009, Paper I). The earliest T-cell progenitors identified in the thymus are referred to as early thymic progenitors (ETPs). This population possesses T, NK and myeloid potential and displays reduced B-cell potential (Allman *et al*., 2003, Bell and Bhandoola, 2008, Wada *et al*., 2008). In support of the thymus being seeded by a precursor with combined myeloid and T-cell potential, such a bi-potent population was recently demonstrated at the single cell level in fetal thymus (Luc *et al*., 2012). Since the ETPs possess myeloid potential, they are postulated to arise from a progenitor pool upstream of the CLP compartment. However this progenitor with combined myeloid and T-lymphoid potential has been questioned. Lineage tracing experiments where cells with a history of IL7r-expression are labeled, indicated that the majority of early T-cell progenitors, despite the majority not expressing *Il7r* mRNA, had a history of Il7r expression in support for the CLP as a source of *in vivo* thymus settling cells (Schlenner *et al*., 2010). Throughout the years, several laboratories have tried to identify the earliest T-cell progenitors with contradictory results (reviewed by Schlenner and Rodewald, 2010 and Zlotoff and Bhandoola, 2011). In sum, our understanding of early T-cell progenitors in the bone marrow is far from complete. Even though a fraction of the CLP compartment possesses T-cell potential its role as an *in vivo* steady-state source for thymus settling cells remains debated.

In addition to an ability to generate B and T-lineage cells, the early CLPs can give rise to NK-cells both *in vitro* and *in vivo* (Welner *et al*., 2009, Paper I). Recently, using ID2-reporter mice, an elegant study identified two populations of NK-precursors denoted pre-pro NKa and pre-pro NKb (Carotta *et al*., 2011). The pre-pro NKa population could potentially represent a lineage restricted progenitor directly downstream of the $LY6D^-$ CLPs since both populations expressed IL7r, SCA1 and KIT at similar levels. Furthermore, $LY6D^-$ CLPs express higher levels of *Id2* than $LY6D^+$ CLPs (Paper II). Independently, a population termed pre-NKP was identified and was suggested to be downstream of the CLPs (Fathman *et al*., 2011). These populations share some surface markers and characteristics, but their exact relationship with the CLP and each other remains to be resolved.

A physiological CLP contribution to the dendritic cell (DC) lineages remains elusive. The DCs are a heterogeneous group of cells with subsets suggested to originate from both early myeloid and lymphoid pathways (reviewed by Belz and Nutt, 2012). Similar as CLPs, DCs rely on FLT3 signaling during development. CLPs have been shown to generate $CD11C^+$ cells both *in vitro* and *in vivo* (Izon *et al*., 2001, Fancke *et al*., 2008). Using the previously mentioned RAG1 fate mapping approach, some DC subsets were labeled, suggesting that these cells are derived through a RAG1 expressing cell stage (Welner *et al*., 2009). In addition to the need of FLT3 signaling, macrophage colony stimulating factor (MCSF) can stimulate DC development and the MCSF-receptor (CD115) is expressed at low levels on the surface of the CLP population

(Fancke *et al*., 2008). Thus this data indicates that a part of the CLP compartment can respond to two key cytokines necessary for DC development. Further CLPs stimulated *in vitro* with ligands for the Toll-like receptors 2 and 4 preferentially generated $CD11C^+$ dendritic cells (Nagai *et al*., 2006).

The CLP population was originally described as lacking myeloid potential (Kondo *et al*., 1997). However, although not as robust as in upstream progenitors like the LMPP, some restricted myeloid potential has been demonstrated to remain in CLPs and $B220^+$ CLP-like populations (Balciunaite *et al*., 2005, Rumfelt *et al*., 2006, Paper I).

Although current literature clearly indicates that B-cell development occurs through a CLP intermediate, alternative routes have been described and question whether the CLP is the only route for B-cell differentiation. Within the LSK^- population, cells with similar characteristics as the CLPs have been identified (Harman *et al*., 2008, Kumar *et al*., 2008). Some of these LSK^- cells exhibited surface expression of both FLT3 and IL7r and lymphoid restriction (Harman *et al*., 2008, Kumar *et al*., 2008).

In summary, the current literature suggests that the CLP compartment contains cells that can give rise to all the lymphoid lineages. It is likely a major source of downstream B-cells and contains cells already committed to the B-cell lineage. Current controversies regarding the contribution of the CLPs (as well as other progenitor populations) to specific lineages most likely arise from the use of similar but not completely identical cell isolation protocols as well as *in vitro* and *in vivo* assays. Further there is a need to distinguish between cell fates still experimentally possible and likely *in vivo* physiological cell fates. In the end, the importance of proper and optimal isolation of progenitor cells can not be stressed enough. Cell isolation is the fundamental basis of all these experiments. Thus, the quality of the data is directly limited by the quality of the isolation. In addition, the age, strain and sex of the research animals might affect surface marker expression and assay read out.

B220 expression on early lymphoid progenitors

Experiments in the early 1980s identified the surface marker B220 as being expressed on bone marrow B-cells (Coffman and Weissman, 1981). Combining B220 expression with the surface markers CD43 (S7), BP1 and HSA (CD24) allowed for the identification and separation of several subsets of early B-cell progenitors before the onset of surface immunoglobulin (Ig)-expression (Hardy *et al*., 1991). In this scheme the earliest identifiable B-cell progenitor population was denoted as fraction A (fr. A) or pre-pro B cells (Hardy *et al* 1991, Philadelphia nomenclature). In parallel, an alternative classification model "The Basel nomenclature" was developed (summarized by Osmond *et al*., 1998). These developmental schemes were further refined through the addition of CD93 (AA4.1) as a marker to segregate for the earliest B-cell progenitors (Li *et al*., 1996). However, subsequent experiments demonstrated the presence of B220 on the cell surface of non B-lineage cells and the bone marrow $B220^+CD43^+$ populations were

shown to be highly heterogeneous with regard to the potential to generate B-cells, limiting the use of $B220^{+}CD43^{+}$ as markers to define early B-cell progenitors (Rolink *et al*., 1996, Nikolic *et al*., 2002, del Hoyo *et al*., 2002, Balciunaite *et al*., 2005).

Since this early scheme for identification of B-cell progenitors relied on a different set of markers than what was used for the identification CLPs, fully reconciling the two schemes was initially technically challenging. Recent advances in flow cytometry have allowed for combining CLP markers (KIT, SCA1, IL7r and FLT3) with classical fr. A/pre-pro B cell markers (B220, CD43 and AA4.1) in addition to an extensive array of lineage markers (MAC1, GR1, TER119, CD3ε, NK1.1, LY6C, CD11C, CD19). These studies illustrated that CLPs and fr. A/pre-pro B cells displayed the same surface phenotype with the exception of low expression of B220 on what classically was referred to as fr. A/pre-pro B cells (Rumfelt *et al*., 2006, Inlay *et al*., 2009, Paper I). Thus, fr. A/pre-pro B cells could be viewed as $B220^{+}$ CLPs (Rumfelt *et al*., 2006, Inlay *et al*., 2009, Paper I). One could then envision that the $B220^{+}$ CLPs would represents the more B-lineage restricted CLPs. But the picture is complex since basing the analysis on expression of a λ5 reporter gene, it became obvious that $B220^{-}\lambda5^{+}$ cells were found to be *in vitro* B-cell committed (all $\lambda5^{+}$ CLPs are $LY6D^{+}$) (Mansson *et al*., 2008). Furthermore a $B220^{+}$ CLP-2 population displayed robust *in vitro* and *in vivo* T-cell potential (Martin *et al*., 2003).

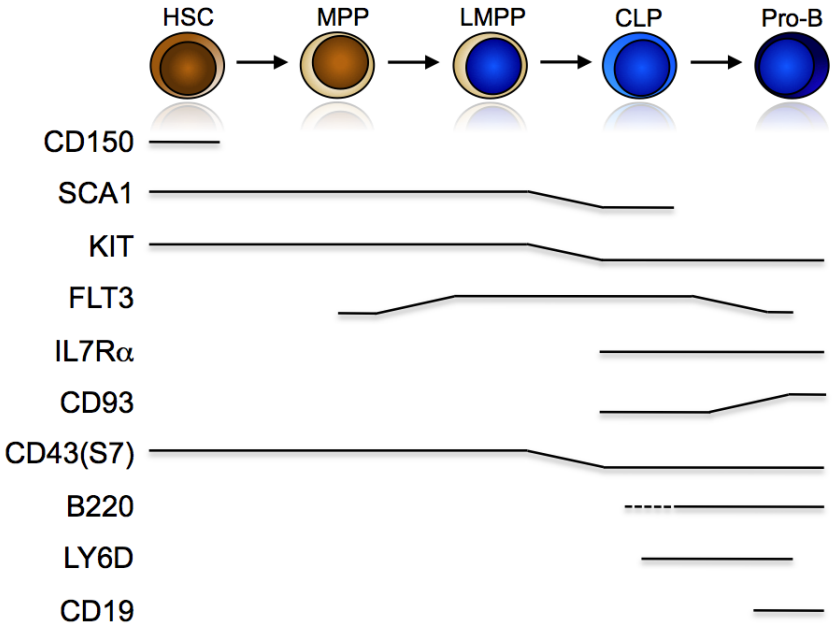

FIGURE 2. Surface markers used to distinguish early hematopoietic progenitor populations. HSC; Hematopoietic stem cell, MPP; Multipotent progenitor, LMPP; Lymphoid primed multipotent progenitor, CLP; common lymphoid progenitor, Pro-B; committed B-cell progenitor.

Using expression of the λ5 reporter it appears as if $B220^+$ cells within the CLP compartment are enriched for B-lineage biased cells, although B220 expression neither correlats directly with B-cell commitment nor a specific developmental stage in B-cell development (Paper I). However, combining B220 with LY6D (most but not all $B220^+$ CLPs are also $LY6D^+$) could allow for segregation of a "true" B-linage biased progenitors. The $B220^+LY6D^+$ CLP/fr A. population most likely represents the direct population upstream the first $CD19^+$ B-cells, since a) the CLP and fr. A populations have overlapping surface phenotypes except for B220 expression, b) the $CD19^+KIT^+/CD43^+$ (often referred to as pro-B cells) express low levels of the CLP markers FLT3 and IL7r and c) about 50% of the pro-B cells express LY6D before surface expression is lost in pre-B cells (Welinder *et al*, unpublished observations).

Bone marrow B-lymphoid development beyond commitment

As the CLPs differentiate along the B-cell pathway, the next discretely defined developmental stage generated is referred to as pro-B cells. These cells display surface expression of CD19 together with KIT and CD43 while still lacking IgM and IgD expression (summarized in Osmond *et al*., 1998). To generate a functional B-cell receptor (antibody) an ordered process called V(D)J-recombination takes place as B-cell development progresses. While $IgH_{D\text{-}J}$ rearrangement is initiated in the CLP compartment, it is completed in pro-B cells and subsequently $IgH_{V\text{-}DJ}$ rearrangement is initiated (Ehlich *et al*., 1994). A successfully rearranged and assembled heavy chain will come together with the surrogate light chains λ5 and VpreB and the Ig-associated proteins CD79a and CD79b encoded by the *Mb1* and *B29* genes respectively to form the pre-B cell receptor complex (pre-BCR). Deposition of the pre-BCR at the cell surface will initiate antigen independent signaling cascades driving the cells to a highly proliferative pre-B cell state (reviewed by Melchers, 2005 and Geier and Schlissel, 2006). Upon attenuation of the pre-BCR signaling, which causes cessation of proliferation, the Rag1/2 genes are re-induced to allow for $IgL_{V\text{-}J}$ rearrangement. Successfully rearranged IgL will replace λ5 and VpreB and together with the IgH form a B-cell receptor (BCR). Deposition of the BCR on the cell surface demarcates the generation of surface IgM^+ immature B-cells (reviewed by Hardy and Hayakawa, 2001). The immature B-cells will then pass through negative selection, where autoreactive cells are depleted or allowed to undergo receptor editing (allowing the formation of a new BCR). B-cells passing this checkpoint exit the bone marrow and migrate to the spleen and secondary lymphoid organs. Here they further mature to form an integral part of the immune system (Hardy and Hayakawa, 2001).

THE B-LINEAGE REGULATORY NETWORK

Identifying network factors promoting the B-cell fate

During the 1990s, a series of knock-out mouse models were generated that upon characterization seemed to more or less completely lack B-cells. These included the knock-outs of the transcription factors PU.1, IKAROS, E2A, BSAP (PAX5) and EBF1 (Scott *et al*., 1994, McKercher *et al*., 1996, Georgopoulos *et al*., 1994, Zhuang *et al*., 1994, Bain *et al*., 1994, Urbanek *et al*., 1994, Lin and Grosschedl, 1995). In addition, the necessity for proper IL7 signaling was established (Peschon *et al*., 1994, von Freeden-Jeffry *et al*., 1995). More recently FOXO1 has been added to the list of transcription factors crucial during early B-cell development (Dengler *et al*., 2008).

The generation of these mouse strains and the characterization of them represent state of the art science that created the foundation for our current understanding of regulatory events in early B-cell development. The later addition of more surface markers and development of novel technologies have enabled us to order these factors into a regulatory network that promote and drive B-lymphoid development.

B-lineage priming

Mice lacking expression of PU.1 die at embryonic day 18.5, with severely reduced numbers of B, T and myeloid cells, whereas MkE development remains largely unaffected (Scott *et al*.,1994). This observed developmental block would indicate a necessity for PU.1 activity at a common developmental stage such as the LMPP or alternatively in early lineage specific progenitor cells. Previously, it has been shown that PU.1 can interact with and thereby prevent the ability of GATA1 to drive cells towards an erythroid cell fate (Rekhtman *et al*., 1999, Zhang *et al*., 2000). Thus, PU.1 appears to actively promote cell fate choices in uncommitted progenitors. Recently it has been shown that PU.1 directly regulates the expression of *Flt3*, hence being essential for the proper formation of LMPPs (Carotta *et al*., 2010). In support for a mechanistic role of PU.1 in a common progenitor population, experiments applying increasing levels of PU.1 promoted macrophage development at the expense of B-lymphoid development (DeKoter and Singh, 2000). Furthermore, PU.1 has been shown to be involved in dictating macrophage and B-lymphoid cell fates by laying down a proper epigenetic landscape and to associate with cell type specific factors (Heinz *et al*., 2010). Hence, careful regulation of PU.1 levels and activity is essential to generate early lymphoid progenitors.

PU.1 positively autoregulates its own gene expression potentially driving cells towards a myeloid fate (Okuno *et al*., 2005, Leddin *et al*., 2011). In order to break this regulatory circuit and allow for lymphocyte development, GFI1 has been suggested to directly interact with and repress the *Sfpi1* promoter (the gene encoding for PU.1) (Spooner *et al*., 2009). This regulatory loop needs to be delicately balanced since PU.1 participates in the regulation of *Il7r* expression which is necessary for the developmental progression from the LMPP stage to the CLP stage (DeKoter *et al*., 2002, Dakic *et al*., 2005). However, deterministic actions correlated to PU.1 levels have been questioned. Reporter mice constructed to report PU.1 transcription without altering *PU.1* mRNA expression levels (through the insertion of ires-GFP downstream in the *Sfpi1* locus) showed similar levels of GFP expression in uncommitted hematopoietic progenitors as well as in the earliest myeloid and lymphoid progenitor populations and conditional deletion of PU.1 in adult progenitors resulted in enhanced generation of granulocyte-like cells (Nutt *et al*., 2005, Dakic *et al*., 2005).

Another transcription factor suggested to be involved in lymphoid specification is IKAROS. Bone marrow ablated for IKAROS expression lack B-cells and exhibits limited T-cell development while erythroid and myeloid cell lineages are present (Georgopoulos *et al*., 1994, Wang *et al*., 1996). Hence these animals display a phenotype resembling that observed in PU.1 knock-out animals. PU.1 is expressed in IKAROS deficient cells and *Ikaros* mRNA can be detected in PU.1 deficient progenitor cells, suggesting that these factors do not initiate each other's expression (Scott *et al*., 1994, Spooner *et al*., 2009). Further in line with a PU.1-like phenotype, IKAROS deficient mice lack detectable LMPPs in the bone marrow and IKAROS has been implicated in the regulation of the *Flt3* gene (Ng *et al*., 2009). However, upon crossing IKAROS ablated mice with reporter mice expressing GFP under the regulatory regions of the *Ikzf1* locus, progenitor cells with deficient MkE potential resembling LMPPs could be identified (Yoshida *et al*., 2006). These LMPPs lacked proper lymphoid priming and could not differentiate further along the B-lymphoid pathway (Yoshida *et al*., 2006).

In terms of promoting B-lineage priming, IKAROS directly regulates *Gfi1* thereby lowering PU.1 levels (Spooner *et al*., 2009). In addition, IKAROS binds specific *Sfpi1* regulatory elements in myeloid and lymphoid lineages, thus both directly and indirectly regulate PU.1 activities allowing for lymphoid differentiation (Zarnegar and Rothenberg, 2012). Further IKAROS is involved in regulating the expression of an extensive set of lymphoid and myeloid related genes (Ng *et al*., 2009). In addition to promoting cell fates, another line of action for IKAROS in lymphoid priming is to suppress the "stemness" gene signature in precursors cells to support differentiation (Ng *et al*., 2009).

Hence both PU.1 and IKAROS participate at the very earliest progenitor stages where B-lineage priming is first initiated.

B-lineage specification & commitment

The transcription factors E47 and E12 (commonly referred to as E2A), both encoded by the *tcf3(tcfe2a)* gene, are indispensable for development of normal numbers and proper lineage priming in LMPPs (Dias *et al*., 2008). In contrast to PU.1 and IKAROS deficient MPPs which cannot differentiate further, CLPs are present in the absence of E2A. These CLPs are severely reduced in numbers and display reduced surface expression of IL7r (Borghesi *et al*., 2005, Inlay *et al*., 2009, Paper II). Further, no expression of LY6D can be detected on the surface of E2A deficient CLPs, suggesting that the developmental block occurs prior to B-cell specification and commitment (Inlay *et al*., 2009, Paper II). The lack of B-cell commitment in these CLPs is striking, since E2A-deficient progenitors are multipotent and can reconstitute the T and NK lineages as well as erythroid-myeloid lineages even after weeks of B-cell promoting cultures and despite the initiation of D_H-J_H rearrangement in these cultures (Ikawa *et al*., 2004). Interestingly, a report where the roles of E47 and E12 were separately analyzed revealed that early B-lymphoid priming and specification was fulfilled by E47 and that E12 is dispensable for B-cell commitment (Beck *et al*., 2009).

E2A belongs to the E-protein family, a basic helix-loop-helix (HLH) transcription factor family with the additional family members HEB and E2-2 (reviewed by Kee, 2009). The E-proteins hetero- or homodimerize in order to bind DNA and the protein activity can be inhibited by Inhibitor of DNA binding (ID) proteins (Kee, 2009). E2A is the best characterized member, in part due to HEB and E2-2 deficiencies being associated with neonatal lethality (Zhuang *et al*., 1996). The generation of transgenic mice carrying floxed HEB/E2-2 alleles has circumvented this issue and unique roles for HEB in NKT-cell development and E2-2 in pDC development have been described (Bergqvist *et al*., 2000, Wojciechowski *et al*., 2007, D'Cruz *et al*., 2010, Cisse *et al*., 2008). Although possessing unique functions, redundancy and dose dependency among the family members has been documented (Zhuang *et al*., 1996, Zhuang *et al*., 1998, Seet *et al*., 2004). This concept was further supported by a report where the roles of HEB were carefully examined in early hematopoietic progenitor populations (Paper II). Compared to E2A deficiency, ablation of HEB in early lymphoid progenitor populations results in a similar but milder phenotype with a partial block in the transition to $LY6D^+$ CLPs (Paper II).

What then is the underlying cause of the B-cell developmental block observed in E2A/HEB deficient CLPs? Early reports suggested that the transcription factor early B-cell factor 1 (EBF1) acts downstream of E2A in the transcriptional hierarchy. Therefore it could be the key target of E2A activity needed for continued development towards the B-cell lineage. This was supported by the findings that a) *Ebf1* expression cannot be detected in E2A knock out cells, b) ectopic expression of E2A can induce *Ebf1* expression and c) expression of *Ebf1* partially circumvents the B-cell developmental block in the absence of E2A (Bain *et al*., 1997, Kee and Murre, 1998, Seet *et al*., 2004).

Within the hematopoietic system, EBF1 is uniquely expressed in the B-lymphocyte lineage, except in plasma cells (Hagman *et al*., 1993). The essential need for EBF1 during B-cell specification and commitment is further stressed by the finding that enforced expression of EBF1 can not only rescue B-cell development in E2A deficient cells but also partially rescues the developmental arrest observed in IKAROS, PU.1 and IL7/IL7r deficient progenitor cells (Reynaud *et al*., 2008, Medina *et al*., 2004, Dias *et al*., 2005, Kikuchi *et al*., 2005). Once expressed, E2A and EBF1 have been suggested to synergistically activate a set of critical early B-cell genes such as *λ5*, *VpreB*, *Mb1*, *B29* and *Pax5* (Sigvardsson *et al*., 1997, O'Riordan and Grosschedl, 1999, Sigvardsson, 2000, Lin *et al*., 2010). Recent EBF1 and E2A chromatin immunoprecipitation assays followed by genome wide deep sequencing (ChIPseq) permitted further insight into how this combinatorial action is achieved (Lin *et al*., 2010, Treiber *et al*., 2010). These studies found collaborative binding of these two factors to be enriched in regulatory regions of genes expressed in pro-B cells (Lin *et al*., 2010, Treiber *et al*., 2010). Hence, lack of EBF1 expression in E2A deficient progenitors emerged as a potential explanation for the developmental block. However, when EBF1 deficient lymphopoiesis was assessed in detail, $LY6D^+$ CLPs were found to be present in close to normal numbers (Zandi *et al*., 2008, Tsapogas *et al*., 2011, Vilgos *et al*., 2012). Although these $LY6D^+$ CLPs lacked B-lineage restriction and expression of the B-cell gene program the developmental arrest seemed to be downstream of the phenotype observed in the absence of E2A (Tsapogas *et al*., 2011).

To further explore the mechanisms behind E2A mediated initiation of B-cell specification, sorted CLPs lacking E2A were profiled for gene expression signatures. Surprisingly few genes with a B-lymphoid connection were affected (Paper II). Interestingly, the transcription factor FOXO1 was included among the genes whose expression was downregulated. Previous studies have demonstrated critical roles for FOXO1 in B-cell differentiation (Dengler *et al*., 2008). FOXO1 has also been extensively characterized as a modulator of the Rag-genes and timely rearrangements of the IgH and IgL chains (Dengler *et al*., 2008, Amin and Schlissel, 2008, Herzog *et al*., 2008, Alkhatib *et al*., 2012, Schultz *et al*., 2012, Ociai *et al*., 2012). In addition, genome wide occupancy of FOXO1 in pro-B cells identified preferential binding of FOXO1 in proximity to E2A in putative regulatory regions (Lin *et al*., 2010). Interestingly, these FOXO1 bound elements were not highly enriched for EBF1 occupancy suggesting that FOXO1:E2A and EBF1:E2A mainly act at separate regulatory elements (Lin *et al*., 2010). Since E2A and FOXO1 are active prior to EBF1, one can speculate that these FOXO1:E2A control elements are already activated during the earliest stages of B-cell specification (Lin *et al*., 2010). Finally, a role for FOXO1 during B-cell commitment was established when FOXO1 deficient bone marrow was shown to exhibit a developmental defect and accumulation of cells in the $LY6D^+$ CLP/fr. A (Paper III).

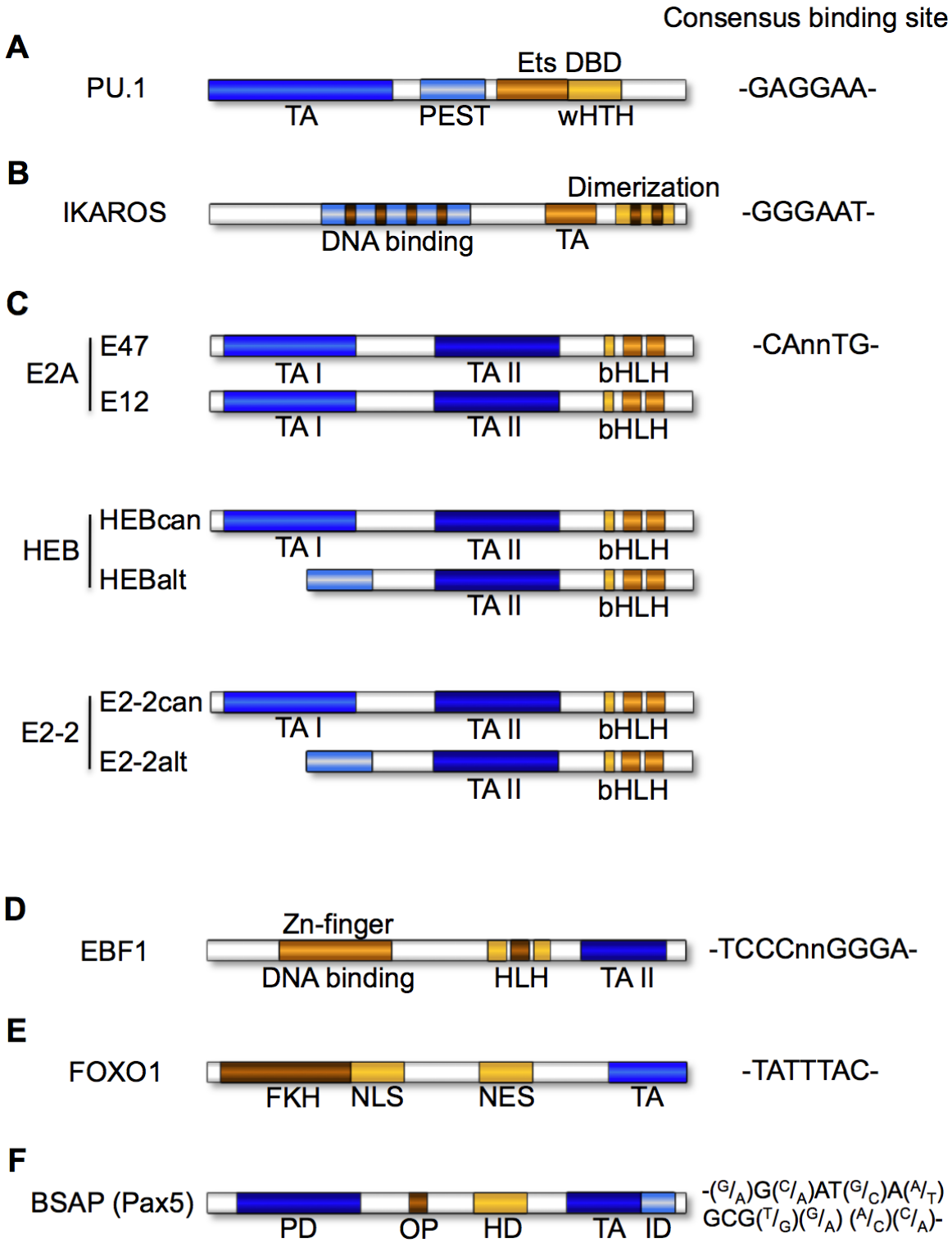
Consensus binding site
A
PU.1
Ets DBD
TA
PEST
wHTH
-GAGGAA-
B
IKAROS
Dimerization
DNA binding
TA
-GGGAAT-
C
E2A
E47
E12
TA I
TA II
bHLH
-CAnnTG-
HEB
HEBcan
HEBalt
E2-2
E2-2can
E2-2alt
D
EBF1
Zn-finger
DNA binding
HLH
TA II
-TCCCnnGGGA-
E
FOXO1
FKH
NLS
NES
TA
-TATTTAC-
F
BSAP (Pax5)
PD
OP
HD
TA
ID
-(G/A)G(C/A)AT(G/C)A(A/T)
GCG(T/G)(G/A) (A/C)(C/A)-

FIGURE 3. Schematic drawings of key transcription factors, their functional domains and major splice variants identified. Proteins and domains are not drawn to scale.
(**A**) PU.1: A N-terminal transactivation domain (TA) is followed by a PEST-domain and the Ets domain. The Ets domain is responsible for DNA binding and protein:protein interactions and includes a winged helix loop helix region (wHLH).
(**B**) The *Ikzf1* gene encodes eight isoforms of IKAROS, depicted here is the largest, Ik-1. Darker brown regions indicate the location of zinc finger domains involved in DNA-binding and protein:protein dimerization. Located in between these regions is the transactivation domain (TA).
(**C**) The E-protein family consists of E2A HEB and E2-2. The two isoforms of E2A (differing in the basic region) is generated through alternative splicing. In contrast, the isoforms of both HEB and E2-2 are generated through alternative promoter usage. The helix-loop-helix (HLH) region mediates protein:protein interactions and the basic (b) region is required for DNA-binding at E-box sites. All the E-proteins share a great degree of sequence overlap in the bHLH region and the two upstream activation domains (AD).
(**D**) DNA-binding by EBF1 is mediated by a Zn finger coordinating domain while the helix loop helix (HLH) promotes protein dimerization. The C-terminal (blue box) area contains the transactivation domain (TA).
(**E**) FOXO1 contains the following domains (in order from the N-terminal), a forkhead DNA binding domain (FKHR), followed by a nuclear localization signal (NLS), a nuclear exportation sequence (NES) and a C-terminal transactivation domain (TA).
(**F**) B-cell-specific activator protein (BSAP) encoded by the Pax5 gene. A paired box domain (PD) mediates DNA binding, the succeeding octapeptide motif (OP) is implicated in gene activation and repression and is followed by a partial homeodomain (HD) participating in protein-protein interactions and finally a transactivation domain (TA) and an inhibitory domain (ID) both regulating transcription.

Specifically, FOXO1 ablated CLPs initiated, but did not complete D_H-J_H rearrangements and readily developed into $NK1.1^+$ and/or $CD11C^+$ cells *in vitro*, resembling the abnormalities in B-cell development observed in EBF1 deficient bone marrow (Zandi *et al*., 2008, Tsapogas *et al*., 2011, Paper III). In addition, the transcriptional profiles of FOXO1 and EBF1 deficient $LY6D^+$ CLPs were highly overlapping. FOXO1 has previously been suggested to be a direct target of EBF1 activity (Zandi *et al*., 2008, Lin *et al*., 2010). Interestingly, the transcriptome of FOXO1 ablated CLPs displayed reduced *Efb1* expression suggesting that these two factors directly cross regulate each other, generating a feed-forward loop to specify the B cell fate (Paper III).

The timed expression of *Ebf1* appears to be key for initiating downstream events leading to B-cell commitment. Premature activation would be detrimental since ectopic expression of *Ebf1* in multipotent progenitors skews development to the B-cell fate at the expense of other lineages (Zhang *et al*., 2003, Pongubala et al., 2008). As described for the E2A proteins, FOXO1 is active in early multipotent compartments but these factors do not induce *Ebf1* until the CLP cell stage (Semerad *et al*., 2009, Yang *et al*.,

2009, Dias *et al*., 2007, Tothova *et al*., 2007). E2A and FOXO1 are by no means the sole factors regulating *Ebf1*. The identification of two *Ebf1* promoter regions revealed a delicate interplay between factors regulating *Ebf1* expression. The distal (α) promoter is regulated by STAT5, E2A and EBF1 and the proximal (β) promoter by PAX5, PU.1 and ETS1 (Smith *et al*., 2002, Roessler *et al*., 2007). Hence this promoter analysis identified a cross regulatory loop between EBF1 and PAX5.

Since EBF1 autoregulates its own expression, inhibiting EBF1 at the protein level indirectly suppresses *Ebf1* transcription. Reduced DNA binding by EBF1 is induced by NOTCH-signaling (Smith *et al*., 2005). The exact mechanism remains elusive, but post translational modifications leading to degradation or outcompeting by NOTCH intracellular DNA-binding partner CSL (which has overlapping DNA-binding sequence with EBF1) has been proposed (Smith *et al*., 2005). In addition, the early hematopoietic zinc finger protein Znf521 has been suggested to modulate developmental progression by binding and antagonizing EBF1 (Mega *et al*., 2011). Recently, genome wide chromosome conformation capture analysis revealed that the *Ebf1* locus is repositioned from a repressive to an active chromatin state during differentiation from multipotent to committed B-cells. This data indicate that factors actively changing the topology of chromatin compartments have fundamental roles in promoting *Ebf1* activation and thereby controlling B-cell differentiation.

STAT5 is a mediator of IL7-signaling (Goetz *et al*., 2004). The notion from the EBF1 promoter analysis that constitutively active STAT5 could activate the α promoter further added to the reports suggesting that *Ebf1* is directly regulated by IL7-signaling (Dias *et al*., 2005, Kikuchi *et al*., 2005). Hence linking IL7-signaling as actively promoting the B-cell fate. B-cell development in the absence of IL7-signaling is blocked in the transition from $LY6D^-$ to $LY6D^+$ CLPs (Tsapogas *et al*., 2011). During downstream B cell development, IL7 signaling mainly induces survival and proliferation and prevents the premature rearrangement of the IgL chains, whereas a lineage instructive role in uncommitted progenitors is debated (reviewed by Milne and Paige, 2006, Johnson *et al*., 2008, Malin *et al*., 2010). T-cell development is disturbed in IL7-signaling deficient mice, however this can partially be circumvented by transgenic expression of *Bcl2*, suggesting a permissive role in lymphocyte development (Akashi *et al*., 1997, Kondo *et al*., 1997, Marakovsky *et al*., 1997). However, deploying the same strategy, B-cell development from IL7 deficient $LY6D^-$ CLPs could not be rescued arguing for the possibility of a more direct role in orchestrating the B-lineage fate (Kondo *et al*., 1997, Marakovsky *et al*., 1997, Sigvardsson lab, unpublished observations).

B-lineage commitment & fidelity

Once activated, E2A, EBF1 and FOXO1 will in concert induce expression of the transcription factor *Pax5* (Pongubala *et al*., 2008, Decker *et al*., 2009, Lin *et al*., 2010, Treiber *et al*., 2010, Paper III). Progenitor cells deficient of PAX5 can generate $\lambda5^+LY6D^+$ CLPs but fail to develop further into $CD19^+$ B-cells (Urbanek *et al*., 1994, Zandi *et al*., submitted manuscript). However, it has been challenging to pinpoint to the precise block in B-cell development since CD19 expression relies on PAX5 activity (Kozmik *et al*., 1992). PAX5 ablated cells express basal levels of several B-lineage associated factors and can D_H-J_H rearrange and induce proximal V_H-D_HJ_H rearrangement of the IgH locus (Nutt *et al*., 1997, Fuxa *et al*., 2004, Zandi *et al*., submitted). All together, these results position PAX5 downstream of E2A, EBF1 and FOXO1 in the transcriptional hierarchy. Although properly B-cell specified, PAX5 deficient cells lack normal B-lineage commitment and retain (even after prolonged culture in B-cell promoting conditions) the ability to give rise to cells of multiple other lineages (Nutt *et al*., 1999, Rolink *et al*., 1999). Strikingly, even deactivation of *Pax5* in committed progenitors allow for dedifferentiation and development into a non B-cell lineage fate (Mikkola *et al*., 2002, Cobaleda *et al*., 2007). Taken together, this data indicates that sustained PAX5 activity is essential to maintain fidelity to the B-lineage fate. Like *Ebf1*, *Pax5* expression is exclusive to the B-lineage branch of the hematopoietic tree and several B-lineage associated transcription factors participate in the regulation of the *Pax5* locus (Fuxa *et al*., 2007, Decker *et al*., 2009). Detailed analyses of target genes has suggested that PAX5 share B-cell target genes with E2A and EBF1 (Sigvardsson *et al*., 2002, Schebesta *et al*., 2007, Treiber *et al*., 2010). *Ebf1* expression in PAX5 deficient cells is sufficient to promote these cells to $CD19^+$ B-cell progenitors (Pongubala *et al*., 2008). This suggests the *Ebf1* promoting function to be one of the key roles for PAX5, effectively creating a stable autoregulatory loop.

Although PAX5 and EBF1 are tightly wired in promoting specification and commitment, they seem to act in both overlapping and parallel circuits since comparison of gene expression patterns derived from PAX5 and EBF1 deficient CLPs revealed differential sets of target genes (Zandi *et al*., submitted manuscript). In further support for non-redundant roles, *in vivo* ectopic *Pax5* expression cannot compensate for the loss of EBF1 in EBF1 deficient progenitors nor can *in vivo Ebf1* expression rescue the inverse situation (Vilagos *et al*., 2012).

However the issue of commitment is more complex than simply just inducing and maintaining a B-lineage specific program of gene expression. For stable commitment, alternative cell fate options must actively be prevented and permanently closed. This is manifested in "EBF1-rescued" IKAROS deficient $CD19^+$ B-cells, where despite expressing normal levels of *Ebf1* and *Pax5* these cells maintain myeloid lineage potential and aberrant myeloid lineage priming (Reynaud *et al*., 2008).

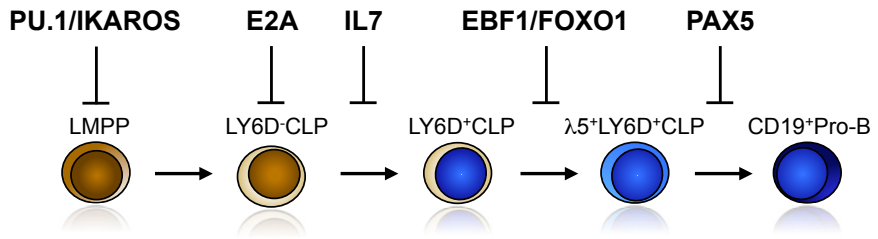

FIGURE 4. Schematic representation of early B-lymphopoiesis displaying B-lineage developmental arrests observed in mice lacking indicated factor.

Suppressing non B-lineage cell fates

Several of the factors in the B-cell promoting network act through dual roles, participating both in initiation and enhancement of expression of B-lineage associated genes in addition to restricting and suppressing genes associated with other cell lineages.

During the transition from the LMPP to the CLP stage, cells largely lose their myeloid potential. As mentioned above, IKAROS deficient progenitors are unable to progress to the CLP stage in addition to being incapable of restraining gene signatures associated with myeloid cell lineages. Hence, indicating a role for IKAROS in dictating the lymphoid versus myeloid cell fate (Yoshida *et al*., 2006, Reynoud *et al*., 2008, Ng *et al*., 2009). Further, E2A deficient MPPs and LMPPs seem to preferentially adopt a myeloid cell fate upon culturing, implicating E2A in myeloid restriction (Dias *et al*., 2008). This ability was later assigned to the N-terminal transactivation domain of E2A (Bhalla *et al*., 2008). Since IL7 signaling is initiated during the transition from LMPPs to LY6D$^-$ CLPs, one could hypothesize that this would have a critical role in suppressing myeloid cell fate options. However, no such mechanism appears to exist (Tsapogas *et al*., 2011). Like IKAROS knock-out cells, PAX5 deficient B-cell progenitors possess the ability to differentiate into myeloid cells and PAX5 can directly suppress some myeloid associated genes including *Mcsfr* (Tagoh *et al*., 2004, Tagoh *et al*., 2006). However, LY6D$^-$ CLPs do not express *Pax5* at a significant level, hence the myeloid fate option must be closed in a developmental window occurring before stable *Pax5* expression is established (Paper I). This myeloid suppressing activity of PAX5 could be part of a B-lineage fidelity mechanism rather than actively promoting myeloid versus lymphoid lineage choices. In support of this notion, enforced expression of *Pax5* in progenitors did not disturb myeloid development (Souabni *et al*., 2002, Cotta *et al*., 2003). However, applying a similar strategy, ectopic *Ebf1* expression efficiently perturbed myeloid

differentiation and ectopic expression of *Ebf1* in PAX5 deficient cells suppressed their T-lymphoid and myeloid potential and antagonized deviant expression of myeloid-associated genes such as *Cebpα* and *PU.1* (Zhuang *et al*., 2003, Pongubala *et al*., 2008). Further, a substantial fraction of the CLPs express *Ebf1* in line with EBF1 participating in eliminating the residual myeloid developmental potential in this population (Paper I).

Using RAG1-transgenic mice strains (discussed in an earlier section), the NK potential of the CLP compartment was assigned to the $RAG1^{low}$ $LY6D^{-}$ CLP population and this potential is largely lost in $LY6D^{+}$ cells (Welner *et al*., 2009, Paper I). Independent reports have indicated EBF1 as a repressor of the NK-associated *Id2* gene and *Ebf1* heterozygous B-cell progenitors maintain priming of several genes connected to NK development (Pongubala *et al*., 2008, Thal *et al*., 2009, Lukin *et al*., 2011). In further support for a role in suppressing NK-cell development, enforced expression of *Ebf1* efficiently blocked NK development while NK-cell development progressed normally in the presence of PAX5 (Zhuang *et al*., 2003, Cotta *et al*., 2003).

However, PAX5 supports EBF1 in suppressing the T-cell fate since *Pax5* expression efficiently impeded T-cell development (Souabni *et al*., 2002, Cotta *et al*., 2003, Fuxa *et al*., 2004, Treiber *et al*., 2010). Some $LY6D^{+}$ cells still exhibit *in vitro* T-cell potential and a fraction of this population expresses *Pax5* at the single cell level (Paper I). However, expression of *Pax-5* message correlates with λ5 expression and *in vitro* B-cell commitment (Paper I). As a mediator suppressing the T-cell lineage fate, PAX5 has been shown to repress *Notch1* transcription (Souabni *et al*., 2002). Further, both *Mscf* and *Flt3* are suppressed by PAX5, suggesting that PAX5 actively suppresses a DC fate (Tagoh *et al*., 2004, Tagoh *et al*., 2006, Holmes *et al*., 2006). Recently it was shown that the *Flt3* gene lacked proper repression during EBF1-deficient B-cell development, thus again wiring PAX5 and EBF1 action (György et al., 2012).

Since EBF1 and FOXO1 act in concert to promote B-cell commitment, one could envision an active suppressor role for FOXO1. FOXO1 ablated $LY6D^{+}$ CLPs exhibit abnormal expression of some T-lineage related genes such as *Thy1.1, Sca1* and *CD28* (Paper III). In addition a modest increase in GMPs can be observed in the bone marrow, which is in line with the increased output of myeloid cells observed in the periphery (Thotorova *et al*., 2007, Murre lab unpublished observations).

Seeking a place in the B-cell transcriptional network

The previous pages have covered current understanding of the best characterized factors and how they act in concert to orchestrate B-cell development. In addition, several other factors have been identified that participate in promoting early B-cell differentiation. Some of them are well characterized in other cellular contexts or as dysregulated during leukemias. However their roles in the transcriptional network that promote B-cell specification and commitment are less well understood.

The MADS-box transcription factor encoded Mef2c has been implicated in myeloid versus lymphoid lineage choices since Mef2c deficiency results in disrupted lymphopoiesis while myelopoiesis is enhanced (Stehling-Sun *et al*., 2009). Mef2c transcription has been suggested to be regulated by PU.1 but direct downstream B-cell related target genes remain unknown (Stehling-Sun *et al*., 2009).

RUNX proteins have been extensively studied in several developmental systems. During *de novo* motif analyses, putative RUNX sites were found to be highly enriched in close proximity to E2A and/or EBF1 occupancy in pro-B cells (Lin *et al*., 2010, Treiber *et al*., 2010). *Runx1* is expressed in early lymphoid progenitors and has been suggested to functionally cooperate with EBF1 (Maier *et al*., 2004, North *et al*., 2009, Blyth *et al*., 2009, Lukin *et al*., 2010). Conditional inactivation of *Runx1* results in a loss of early lymphoid progenitors. However contradictory to these findings overexpression of *Runx1* was shown to result in a block in B-cell development (Growney *et al*., 2005, Blyth *et al*., 2009). Hence the detailed mechanisms of action for RUNX1 during B-cell differentiation remain elusive.

Miz1 deficient mice exhibit an early B-cell developmental block possibly caused by an impaired response to IL7 signaling (Kosan *et al*., 2010). In line with this, $CD19^{+}$ cell development was partially rescued in Miz1 deficient cells upon combined *Bcl2* and *Ebf1* expression (Kosan *et al*., 2010).

Another factor implicated in promoting B-cell differentiation at the CLP stage is c-Myb (Greig *et al*., 2010). c-Myb is suggested to directly regulate the expression of IL7r, however enforced expression of IL7r did not rescue B-cell development suggesting this factor to be involved in B-cell differentiation at multiple levels (Greig *et al*., 2010).

Bcl11a deficiency results in embryonic lethality. Upon transplantation, Bcl11a ablated progenitor cells did not properly support B-cell development and showed T-cell developmental abnormalities (Liu *et al*., 2003). These cells lacked expression of *Ebf1* and *Pax5* transcripts and consequently did not have the ability to induce the B-cell gene program and suppress T-lineage development. Further ChIPseq analyses identified a putative Bcl11a control element occupied by both E2A and FOXO1 in pro-B cells. All-in-all, these results suggest that Bcl11a is an important member of the B-cell network (Lin *et al*., 2010).

The need for SOX4 in developing B-lymphocytes was established during the 1990s when transplanted SOX4 deficient progenitors failed to properly reconstitute the B-cell lineage (Schilham *et al*., 1996). *Sox4* has been suggested to be activated by IKAROS and displays correlated expression with *Ebf1* in B-cells (Mansson *et al*., 2004, Ng *et al*., 2009). Further, SOX4 binds and activates a λ5-VpreB enhancer during ESC differentiation and thus it appears to actively promote the B-cell fate (Liber *et al*., 2010).

In the B versus T-lineage choice, LRF is actively blocking NOTCH signaling to allow for B-lymphoid development (Maeda *et al*., 2007). Progenitor cells lacking LRF displayed increased activation of several genes downstream of NOTCH and ectopic expression of *Ebf1* could not rescue the phenotype (Maeda *et al*., 2007).

Irf8 is a suggested PAX5 target with established functions during several stages of B-cell differentiation. Specifically IRF8 was shown to activate *PU.1* and *Ebf1* expression and promote the pre-B to mature B-cell transition (Schebesta *et al*., 2007, Lu *et al*., 2006, Wang *et al*., 2008). Interestingly, IRF8 was recently found to bind a *Pax5* enhancer element providing yet another a feed forward loop promoting B-cell lineage identity (Decker *et al*., 2009).

Cells unable to properly contract and rearrange IgH are developmentally blocked at a $CD19^+$ pro-B cell stage. A vast majority of the classical B-cell promoting transcription factors have been implicated in IgH locus contraction and remodeling to facilitate recombination (reviewed in Bossen *et al*., 2012). In addition, several factors with knock out models displaying pro-B cell related developmental blocks are implicated in regulating IgH locus accessibility, contraction and rearrangement. Among these are Ezh2 (Su *et al*., 2003), Foxp1 (Hu *et al*., 2006), YY1 (Liu *et al*., 2007) and the YY1 binding factor Gon4-like (Lu *et al*., 2011). It will be important to ultimately integrate the entire ensemble of the aforementioned factors into a global network to describe the B cell fate in mechanistic terms.

Epigenetic regulation of B-cell differentiation

The chromatin structure and epigenetic status of DNA is closely associated with its transcriptional state. Actively transcribed regions share structural and epigenetic features with other active regions. While a promoter is located adjacent to the TSS, enhancers are regulatory regions that can be both proximally and distally located upstream, downstream or within their target genes (reviewed by Visel *et al*., 2009). Chromatin remodeling complexes (CRCs) are recruited to regulatory elements in order to access and facilitate transcriptional initiation or to repress target genes. Two of the most widely characterized CRCs are the SWI/SNF and NuRD complexes. SWI/SNF protein complexes can directly open up the chromatin through repositioning of nucleosomes thereby making DNA accessible for activation. NuRD complexes are thought to mainly act in a repressive manner through chromatin compaction (reviewed by Ho and Crabtree, 2010). The histones in the nucleosomes as well as DNA can be directly modified. These modifications includ, but are not limited to, direct methylation of cytosines as well as methylation and acetylation of histone lysines (reviewed by Kouzarides, 2007). Several co-factors associated with the CRCs participate in establishing and changing these modifications. Recent reports suggest that enhancers are marked by the acetylation of multiple H3 and H4 residues as well as monomethylation of histone H3 at lysine 4 (H3K4me1). On the other hand, enhancers lack trimethylation of H3K4 (H3K4me3), which is associated with active promoters (Heintzman *et al*., 2009). Hence, reading the epigenome can directly indicate whether a certain promoter or regulatory region is poised, actively transcribed or kept silent and several studies have reported changes in

epigenetic patterns associated with lineage restriction during hematopoietic differentiation (Attema *et al*., 2007, Weishaupt *et al*., 2010, Ji *et al*., 2010).

Knock-out models of components of polycomb repressor complexes (PRC) involved in maintaining methylation patterns has been shown to display lineage differentiation biases. Mice lacking functional expression of *Bmi1*, a component of PRC1, showed increased *Ebf1* and *Pax5* expression and increased numbers of lymphoid progenitors (Oguro *et al*., 2010). The premature expression of *Ebf1* and *Pax5* is likely, in part, caused by a lack of suppression of *Ikaros* in Bmi1 deficient progenitors (Arranz *et al*., 2012). Further, epigenetic marks maintained by the PCR1 component Dnmt1 has been suggested to suppress myeloid-erythroid potential to allow for lymphoid development (Broske *et al*., 2009). Recently it was shown that deficiency of the histone H2A deubiquitinase MYSM1 resulted in an early B-cell developmental block and an underlying factor behind the arrest was suggested to be a lack of proper activation of the *Ebf1* locus (Jiang *et al*., 2011).

The question that arises is whether transcriptional regulators such as transcription factors act to open up the chromatin, thus allowing for epigenetic patterns to change or if changes in chromatin subsequently allow specific transcription factors to access the chromatin? B-lineage associated genes have been shown to have pre-established active marks already in uncommitted progenitors, potentially as a consequence of low levels of expression of lineage associated transcription factors (Walter *et al*., 2008, Mercer *et al*. 2011). Further, a recent report has suggested that the changes in epigenetic patterning can be a consequence of PU.1 acting as a pioneer factor to promote or maintain certain chromatin states and thereby allowing or inhibiting differentiation and lineage choices (Heinz *et al*., 2010). In line with this observation, expressing E47 in E2A deficient cells rapidly induced a change in the H3K4me1 pattern (Lin *et al*., 2010). E2A occupancy is closely linked to enhancer regions and p300, a known co-activator and histone acetyl-transferase, which E2A can directly interact with (Bradney *et al*., 2003, Lin *et al*., 2010, Lin *et al*., submitted). Further, IKAROS has been suggested to perform the majority of its transcriptional effects through interactions with CRCs. In T-lymphocytes, IKAROS is often found associated to NuRD and to lesser degree SWI/SNF-complexes. In line with a transcription factor instructive role, IKAROS was recently shown to directly alter the functions of NuRD complexes (Zhuang *et al*., 2011).

EBF1 has been shown to be able to, in a dose dependent manner, effect localization of a gene to either hetero- or euchromatin (Lundgren *et al*., 2000). In addition, numerous reports have shown that both EBF1 and PAX5 to modulate the chromatin status and potentially recruit CRCs and their coactivators (Zhao *et al*., 2003, Walters et al., 2008, Decker *et al*., 2009, Treiber *et al*., 2010, McManus *et al*., 2011). Their sequential and combinatorial activation of the *Mb1* promoter is a carefully studied example (Maier *et al*., 2004, Gao *et al*., 2009). Chromatin remodeling by FOXO1 is less well characterized, however recombinant FOXO1 can *in vitro* disrupt histone:DNA interactions and other FOX-family factors have been shown to influence higher order

chromatin organization (Hatta and Cirillo, 2007, Scott and Plon, 2005, Yan *et al*., 2006, reviewed by Friedman and Kaestner, 2006).

Taken together, the vast majority of critical transcription factors seem to directly interact with CRCs and thereby influence chromatin status and transcription but exact mechanisms are still to be determined.

SUMMARY AND DISCUSSION OF PAPERS

PAPER I

Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity.

Summary

Previously work performed in the Sigvardsson laboratory has demonstrated that a λ5-reporter could be utilized in order to identify cells committed to the B-lineage prior to the onset of CD19 surface expression (Mansson *et al*., 2008). To further expand upon this finding and to study early B-cell developmental, mice carrying λ5-reporter were bred with RAG1-GFP reporter mice. In mice, carrying both reporters, the CLP compartment and pre-pro B-cells could be subdivided into three distinct populations $GFP^{low}\lambda5^{-}$, $GFP^{high}\lambda5^{-}$ and $GFP^{high}\lambda5^{+}$.

Through *in vitro* assays we could show that these subpopulations constitute of a hierarchal organization where lineage potentials are gradually lost before cells commit to the B-cell lineage. On a single cell level, GFP^{low} cells could give rise to B, T and NK cells whereas the $GFP^{high}\lambda5^{-}$ cells were restricted in their NK-cell potential. Further, while the $GFP^{high}\lambda5^{-}$ still generated both B and T-cells the $GFP^{high}\lambda5^{+}$ displayed a high level of commitment to the B-cell lineage. Through transcriptional profiling of these populations we identified the surface marker LY6D as being expressed in the latter two populations and its upregulation highly correlated with the loss of NK-cell potential.

Discussion and future directions

In a report preceding Paper I by just a few months, Inlay and colleagues reported that the CLP compartment could be divided into two populations based on the surface marker LY6D (Inlay *et al*., 2009). $LY6D^{+}$ CLPs were shown to be more specified towards the B-cell lineage further supporting the conclusions from Paper I. However our *in vitro* data do not fully support B-lineage restriction in the $LY6D^{+}$ CLPs. This discrepancy can be potentially accounted for through methodological choices. Inlay and colleagues relied on bulk transplantations assays while the concussions in Paper I were based on *in vitro* single cell assays.

Assigning lineage potential is a challenging task and depending on the assay applied, the outcome might vary (Riche Ehrlich *et al*., 2010). *In vivo* assays obviously

have the advantage of being *in vivo*. However, they have several limitations that must be considered which raise the question as to how close to a normal *in vivo* setting transplantation experiments actually are? To reach their native environment, transplanted progenitor cells need to express the correct surface receptors in order to migrate to the correct microenvironment. Further, when assaying progenitors lacking long-term reconstitution potential, timing of the read out is critical since various lineages display different half-lives. In addition, the more limited proliferative potential a progenitor displays, the more progenitor cells need to be transplanted to allow for detection of progeny *in vivo*. *In vitro* assays display a different set of limitations. They provide signals from an artificial environment likely sometimes far from an actual physiological condition. Further the quality of the assay will heavily influence the outcome, as will the assays chosen *per se* as different conditions can yield different readouts There are however benefits using *in vitro* assays in that they can be used to study single cells. One should further consider the differences between the physiological steady-state contribution of a progenitor and its lineage potential. Indeed the $LY6D^+$ CLPs might be more likely to generate B-lineage cells *in vivo*, even if they still retain T-cell lineage potential. This is supported by the identification of the developmental block in E2A-deficent mice, where no $LY6D^+$ cells are formed, but all other lymphoid lineages except B-cells are present. Together, the two reports beyond doubt, show that the $LY6D^+$ cells represent a more lineage restricted population *en route* to B-lymphoid development. In addition to LY6D, the CLP compartment displays heterogeneous expression of other surface markers, hence it should be possible to further subfractionate the lymphoid developmental pathway in the future.

Further both Inlay *et al*., 2009 and Paper I address the definition of the fr. A/pre-pro B population and show through multicolor flow cytometry that this fraction of cells correctly is represented by $B220^+LY6D^+$ CLPs. A similar conclusion has been suggested in a previous report in 2006 by Rumfelt *et al*.,.

PAPER II

E2A and HEB Act in Concert to Induce the Expression of FOXO1 in the Common Lymphoid Progenitor

Summary

Together, E2A, HEB and E2-2 constitute the E-protein family. Several lines of evidence suggest a functional redundancy amongst these proteins though they display different patterns of expression. Here we first identify HEB expression in several hematopoietic progenitor populations, which prompted us to further investigate the role of HEB in hematopoietic development. In contrast to E2A-deficient animals, mice with HEB ablated bone marrow showed a normal number of HSCs, MPP and myeloid-erythroid progenitors. Further characterization identified a slight reduction of LMPPs and a partial block in the developmental transition from $LY6D^-$ to $LY6D^+$ CLPs. Thus this transition is affected in a similar manner to what is observed in E2A-deficient mice. Further, *in vitro* differentiation assays revealed that these $LY6D^-$ CLPs had reduced B-cell potential but developed into early T-cells at expected ratios. HEB ablated $LY6D^+$ CLPs exhibited normal D_H-J_H rearrangements and downstream progenitors developed at normal ratios. Transcriptional profiling of E2A and HEB deficient $LY6D^-$ CLPs displayed a substantial overlap, where the B-cell related transcription factor *Foxo1* was one of the down regulated genes.

We further identify an E-box in a 3' putative enhancer element of the FOXO1 locus, which was shown to be relevant for *in vitro* transcriptional activity. All-in-all this suggests that E-proteins may directly regulate *Foxo1* expression and that HEB supports E2A in promoting the B-cell fate in the LMPP and $LY6D^-$ CLP compartment.

Discussion and future directions

HEB has previously primarly been studied in the context of T-cell development, where HEB and E2A act together during developmental checkpoints. In addition a role in pro-B cell maintenance has been described. In Paper II we assign HEB a supporting role to E2A during early B-cell specification. Here, HEB and E2A appear to act in the same pathway. This is supported both by a similar *in vivo* phenotype as well as an overlapping dysregulation of gene expression in ablated mice. The actions of E2A and HEB can simply be explained in terms of E-protein dose dependency. Simplified we can extrapolate one allele of E2A as being equal to two alleles of HEB which would fit mRNA expression levels observed. However, obviously such speculation does not take into account factors such as protein stability and posttranslational modifications.

Several questions pertaining to this study remains to be answered. This includes if the target genes of E2A and HEB are exactly overlapping, if E2A and HEB bind the same E-boxes and if E2A and HEB are binding DNA as homo- or heterodimers? In order to answer these questions we attempted to perform HEB ChIPseq. However with the currently available commercial antibodies raised against HEB we were not able perform ChIPseq on early B-cell progenitors.

Another intriguing question that remains to be resolved is the issue as to what drives the specific B-cell promoting properties of E2A? And further, what is the underlying cause of the developmental block observed in E2A ablated mice? As reported in Paper II *E2A* mRNA levels do not change between the LMPP, LY6D$^-$ and LY6D$^+$ CLP populations to any significant degree, nor is there any remarkable change in the expression of the inhibitors *Id2* and *Id3*. E2A has been suggested to heterodimerize with the bHLH proteins TAL and LYL1 in hematopoietic progenitors (Hsu *et al*., 1991, Miyamoto *et al*., 1996). Both TAL and LYL1 have limited abilities to homodimerize and have been suggested to prefer heterodimerization with E-proteins (Hsu *et al*., 1991, Miyamoto *et al*., 1996). Since E2A had been suggested to drive B-cell differentiation as a homodimer, one could hypothesize that if levels of TAL and LYL1 decline as a cell differentiates, E2A would be allowed to homodimerize and activate B-cell specific targets. However, overexpression of E47 in progenitor does not appear to enhance B-cell development (Pongubala *et al*., 2008). The data in Paper II also opens up a participation of possible heterodimers of E2A and HEB, similarly as observed during T-cell development (Barndt *et al*., 2000).

Our aim was to provide important clues about underlying cause of the developmental block observed in E2A deficient cells. Few genes changed in the microarrays had known B-cell relevance. FOXO1 and EBF1 (discussed in Paper III) are necessary for proper B-cell commitment. However, the developmental block observed cannot be accounted for by the lack of these factors since LY6D$^+$ cells develop in their absence. We cannot rule out that E2A drives the expression of LY6D, however, the LY6D$^-$ compartment is drastically reduced and the transcriptional profiles of E2A and FOXO1/EBF1 ablated CLPs are not overlapping. A previous report has suggested the impaired B-cell development to be, in part, due to a lack of proper IL7-signaling response (Seet *et al*., 2004). Indeed we saw reduced expression of IL7r but the recently characterized IL7-developmental block would argue against this as a sole explanation (Tsapogas *et al*., 2011).

PAPER III

The Transcriptional Regulators, FOXO1 and EBF1, Establish a Feed-Forward Loop to Orchestrate the B cell Fate

Summary

The Murre lab has recently reported genome-wide occupancy of E2A, EBF1 and Foxo1 in pro-B cells demonstrating that FOXO1 is an essential part of the early B-cell regulatory program (Lin *et al*., 2010). This together with our findings in Paper II prompted us to further explore the role for FOXO1 in B-cell specification and commitment. To achieve this objective mice deficient for FOXO1 were characterized. A near complete block in B-cell development was observed at the $LY6D^+$ CLP cell stage. The transcriptome of these $LY6D^+$ cells exhibited a lack of B-cell related genes (normally expressed at this stage of development), including most notably *Ebf1*. Additional analysis of $LY6D^+$ cells ablated for FOXO1 showed striking similarities with EBF1 deficient $LY6D^+$ CLPs. *Foxo1* has previously been shown to be a target of EBF1 activity. Together this data suggests that FOXO1 and EBF1 act to positively cross regulate each other. Upon careful examination of the reported ChIPseq data for FOXO1 and EBF1 we identified putative FOXO1 binding in putative *Ebf1* enhancer regions and vice versa. Further, we generate a global network that is based on transcription factor signatures of FOXO1 and EBF1-deficient B cell progenitors, genome-wide interactions between promoter and enhancer elements involving FOXO1 and EBF1 occupancy. All together this suggests that EBF1 and FOXO1 generate a feed-forward loop, thus, locking cells into the B-cell fate.

Discussion and future directions

In Paper III we are able to pinpoint the importance of FOXO1 in the transcriptional network that promotes B-cell commitment (together with EBF1) in the $LY6D^+$ CLP compartment. In light of previously published data these findings lead us to suggest a model for the transcriptional control of early B-cell development where E2A activate the expression of FOXO1 and EBF1 to specify the B-cell fate. Since uncommitted B-cell primed progenitors already have established a B-cell poised chromatin state, one could hypothesize that once EBF1 and FOXO1 activity reaches a critical level they reinforce each other to a level where they override all other fate options.

The identification of a critical role for FOXO1 at a cellular stage relying on proper FLT3 and IL7 signaling might seem contradictory. The activity of the FOXO protein family is extensively controlled through post-translational modifications

(reviewed by Hedrick, 2009). Among the mechanisms that regulate FOXO activity is PI(3)K-AKT mediated phosphorylation, which results in inactivation through exclusion from the nucleus (Hedrick, 2009). The main action of IL7 signaling at the CLP stage has been suggested to be activation of STAT5 rather than through the activation of AKT (Kikuchi et al., 2005, Åhsberg et al., 2010, Johnson et al., 2008). Thus, it might rather be that FLT3 signaling activates the PI(3)K pathway at this stage of development and until the receptor is downregulated at later developmental stages by PAX5 (Holmes et al., 2006). However modulators of the PI(3)K pathway such as *Pten*, *Blnk* and *Pik3ip1* appears to be regulated by E2A, FOXO1 and EBF1. This suggests that these cells are primed by E2A initially to allow for FOXO1 activity and this is further enhanced by FOXO1 itself once activated.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Från blodstamcell till B-cell

Vårt blod består av flera olika specialiserade typer av celler. Dessa utför allt från transport av syre (röda blodkroppar) och sårläkning (blodplättar) till att utgöra vårt immunsystem (vita blodkroppar). Vårt immunförsvar kommer ständigt i kontakt med virus, bakterier och sjuka celler som måste kännas igen och bekämpas. Därför finns det ett kontinuerligt behov av att bilda nya vita blodkroppar. Nybildningen av dessa sker från blodstamceller i benmärgen. Stamcellerna kan när de delar sig både ge upphov till nya stamceller (självförnyelse) eller till dotterceller som sedan kan ge upphov till alla typer av blodceller. Dottercellerna som bildas går igenom flera utvecklingsstadier där de gradvis specialiseras mot att bli en specifik typ av blodcell. Slutligen är cellen så specialiserad att den bara kan ge upphov en specifik celltyp. Därefter utvecklas de vidare för att kunna utföra sin specifika uppgift. De olika utvecklingsstadierna kan man särskilja med hjälp av proteiner på cellernas yta så kallade ytmarkörer. Vad som styr stamcellerna till att bilda en viss celltyp är ett avancerat samspel mellan proteiner inuti cellen och yttre signaler från proteiner i cellens omgivning. Av stor vikt är transkriptionsfaktorer. När denna grupp av proteiner binder till arvsmassan (DNA) kan de aktivera eller stänga av gener. På detta sätt påverka transkriptionsfaktorerna hur mycket av en viss gen som ska uttryckas och ge upphov till protein och därmed styr hur cellen utvecklas och beter sig.

I den här avhandlingen har vi studerat närmare hur blodstamceller ger upphov till den speciella typ av vita blodkroppar som kallas B-celler. I **Arbete I** har vi identifierat nya utvecklingsstadier av celler som är på väg att bli B-celler. Vi fann att ytmarkören LY6D kunde dela upp ett tidigare beskrivet utvecklingsstadie och att celler med LY6D på cellytan var mer mogna och specialiserade för att bli B-celler. Fortsättningsvis har vi studerat dessa två nya utvecklingsstadier för att förstå hur transkriptionsfaktorer och signaler från den omgivande miljön i benmärgen samverkar för att styra dessa celler till att bli B-celler (**Arbete II-III**). Vi kunde visa att transkriptionsfaktorerna E2A och HEB i de tidiga cellerna som inte har LY6D på cellytan, aktiverar transkriptionsfaktorn FOXO1, som är viktigt för B-cellsutvecklig **(Arbete II)**. I utvecklingsövergången till stadiet där LY6D finns på cellytan aktiverar E2A och FOXO1 en annan transkriptionsfaktor som är kritisk för B-cells utveckling och som kallas EBF1. Slutligen föreslår vi att EBF1 och FOXO1 aktiverar varandra och att denna självförstärkande cirkel resulterar i att cellerna specialiserar sig till den grad att de blir låsta till att fortsätta utvecklas mot att bli B-celler (**Arbete III**).

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