Immunity
Reviews

The Transcriptional Regulation of B Cell Lineage Commitment

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The expression of lineage-associated genes, as well as the survival and expansion of committed B cell progenitors, is controlled by multiple transcriptional regulators and growth-factor receptors. Whereas certain DNA-binding proteins, such as Ikaros and PU.1, are required primarily for the formation of more primitive lymphoid progenitors, other factors such as E2A and EBF1 have more direct roles in specifying the B cell-specific gene-expression program. Further, Pax5 functions to promote B cell commitment by repressing lineage-inappropriate gene expression and reinforcing B cell-specific gene expression. In this review, we focus on recent studies that have revealed that instead of a simple transcriptional hierarchy, efficient B cell commitment and differentiation requires the combinatorial activity of multiple transcription factors in a complex gene regulatory network.

Introduction

Development of a functional immune system requires appropriate regulation of dynamic transcription-factor networks that activate lineage-specific gene expression and restrict the differentiation options of hematopoietic stem cells (HSCs) and their progeny (Laiosa et al., 2006). A description of both the wiring and the logic of these transcriptional networks will be essential for a complete understanding of immune cell development and how alterations in these networks lead to diseases such as immune deficiency, autoimmunity, and cancer. As the sole source of immunoglobulin, B lymphocytes (or B cells) are an essential component of the adaptive immune system, and the cellular and molecular mechanisms that control their development have been under intense scrutiny for the past two decades (reviewed by Hardy et al. [2007] in this issue of *Immunity*). B lymphopoiesis has emerged as one of the leading models for studies of lineage specification (induction of a lineage-specific gene-expression program) and commitment (repression of alternative gene-expression programs) owing to the availability of in vitro culture conditions that support B cell development and the ease by which these cells can be analyzed in vivo. Given these advantages, many of the key regulators of B lymphopoiesis have been identified and complex networks of interactions are beginning to be revealed. In this review, we examine the known components of the transcriptional networks that promote specification and commitment of HSCs to the B cell lineage.

Overview of Early B Cell Development

B cells, like all hematopoietic cells, are produced in a stepwise process from self-renewing HSCs in the fetal liver and postnatal bone marrow. The earliest differentiated progeny of HSCs are multipotent progenitors (MPPs), which have lost the capacity for extensive self-renewal but retain multilineage differentiation potential (Adolfsson et al., 2001). A subset of MPPs expressing high amounts of the tyrosine kinase receptor Flt3 have little erythromegakaryocytic potential but retain lymphoid and other myeloid potential leading to their designation as lymphoid-primed MPPs (LMPPs) (Adolfsson et al., 2005). The LMPP population contains early lymphoid progenitors (ELPs), lymphoid-restricted cells defined by expression of a *Rag1*-GFP reporter (Igarashi et al., 2002; Schwarz et al., 2007). LMPPs or ELPs, in particular those expressing CCR9 or CD62L, function as efficient thymus-seeding progenitors and are the likely precursors to early T lineage progenitors (ETPs), although the precise origin of ETPs remains controversial (reviewed by Bhandoola et al. [2007] in this issue of *Immunity*). ELPs are the precursors of bone-marrow common lymphoid progenitors (CLPs), which give rise to B lymphocytes, natural killer (NK) cells, dendritic cells (DC), and T lymphocytes but lack all myeloid potential when tested in vivo but not in vitro (Balciunaite et al., 2005; Kondo et al., 1997; Rumfelt et al., 2006; Traver et al., 2000). However, in vivo CLPs may be primarily progenitors of B lymphocytes and NK cells (Allman et al., 2003; Harman et al., 2006; Kondo et al., 1997). The first clearly identifiable B cell-specified progenitors arise from CLPs in the bone marrow and are variously termed pre-pro B cells, fraction A, or CLP-2 and can be identified by expression of the B cell-associated marker B220 and activation of many B cell-lineage-associated genes (Gounari et al., 2002; Li et al., 1996; Rumfelt et al., 2006). Cells committed to the B cell lineage can be identified by expression of CD19, a target of the lineage-commitment factor Pax5 (see below).

Lymphoid Specification

Until recently, it was believed that the first step in MPP differentiation resulted in efficient segregation of lymphoid and myeloid potential resulting in CLPs and common

Table 1. Structure and Function of Transcription Factors Implicated in the Networks Controlling B cell Development

Transcription Factor (Gene Symbol)	DNA-Binding Motif (Consensus-Binding Sequence)	Ectopic Expression Phenotype	B Cell Phenotype of Mouse Germline (KO) and Conditional (cKO) Deficiency	Association with Human B Cell Malignancies
PU.1 (*Sfpi1*)	Ets (AGGAAGT)	In PU.1$^{-/-}$ fetal-liver cells graded amounts of PU.1 rescue B cell and macrophage development.	KO: lack fetal B cells and CLPs; cKO: normal B2 and expansion of B1 cells.	Not mutated in B-ALL.
Ikaros (*Ikzf1*)	Zinc finger (TGGGAA)	N.D.	KO: lack all stages of B cell differentiation.	Deletions identified in 17 cases of B-ALL.
E2A (*Tcfe2a*)	bHLH (CANNTG, prefers ACACCTGC)	Induces cell-cycle arrest and apoptosis in T or B cell lines, in the 70Z/3 macrophage line induces B cell-lineage conversion.	KO: block prior to pre-pro-B cells; cKO: reduced survival in pre-B cell lines. Ectopic E protein antagonist: pro-B cell-growth arrest, decreased pro-B cell gene expression.	E2A-Pbx1 and E2A-HLF translocation in approximately 6% of B-ALL, mutation in one case of B-ALL.
Early B cell factor, EBF1 (*Ebf1*)	Zinc knuckle (ATTCCCNNG GGAAT)	Induces B cell differentiation in multipotent progenitors, rescues B lymphopoiesis in PU.1$^{-/-}$, E2A$^{-/-}$ or IL-7R$^{-/-}$ progenitors.	KO: arrest at CLP to pre-pro-B cell transition, no cells with *Igh* gene rearrangement.	Eight cases of B-ALL with monoallelic deletion of the *EBF1* gene, six of these deletions are limited to *EBF1*.
Pax5 (*Pax5*)	Paired domain ($^{A}/_{G}$NCNANT$^{C}/_{G}$A$^{T}/_{A}$ GCG$^{G}/_{T}$A$^{A}/_{G}$T$^{A}/_{A}$A$^{A}/_{C}$)	Impairs T cell development and promotes T cell-lymphoma formation. Variably affects myeloid and erythroid differentiation.	KO: Fetal liver lacks B lineage cells. Adult bone marrow block at pro-B cell stage but have D-J$_H$ but only a few proximal V-DJ$_H$ rearrangements; cKO: required for the maintenance of B cell fate and repression of plasma-cell differentiation.	Copy-number alterations, primarily monoallelic loss in 29.7% of B-ALL. Rearrangements to the IgH in t(9:14) translocation in large diffuse cell lymphoma/ B-NHL.
Aiolos (*Ikzf3*)	Zinc finger (TGGGAA)	N.D.	KO: regulates B cell activation and differentiation to effector stage. Represses λ5 in pre-B cells.	Deletion identified in three cases of pre-B-ALL.
Sox4 (*Sox4*)	HMG-box (CCTTTGAA)	N.D.	KO: lethal at e13.5, pro-B cells fail to expand in IL-7 and few pro-B after fetal-liver transfer into irradiated adults.	N.D.
Lymphoid enhancer factor, Lef1 (*Lef1*)	HMG-box (CCTTTGA/T A/T)	N.D.	KO: decreased pro-B cells in fetal liver and neonatal bone marrow, pro-B cells respond to IL-7 but not Wnt3a.	Deletions identified in three cases of B-ALL.
Bcl11a (*Evi9*)	Zinc finger (GGCCGG)	N.D.	KO: no B lineage cells.	Amplified in approximately 20% of B-NHL.

Table 1. *Continued*

Transcription Factor (Gene Symbol)	DNA-Binding Motif (Consensus-Binding Sequence)	Ectopic Expression Phenotype	B Cell Phenotype of Mouse Germline (KO) and Conditional (cKO) Deficiency	Association with Human B Cell Malignancies
GABP (*gabpa*)	Ets (AGGAAGT)	N.D.	Hypomorphic gene trap allele lethal between E12.5-14.5. Impaired B cell development and *Pax5* expression after fetal-liver transfer into irradiated adults.	N.D.

N.D., not determined; ALL, acute lymphoblastic leukemia; and NHL, non-Hodgkin's lymphoma. The relevant references can be found in the text.

myeloid progenitors (CMPs) (Akashi et al., 2000). However, the identification of LMPPs revealed that the loss of myeloid potential en route to lymphoid specification is a more gradual process and is associated with increasing expression of Flt3 (Adolfsson et al., 2005; Mansson et al., 2007). Given this recent realization, our understanding of the mechanisms driving specification of LMPPs from MPPs is limited compared to what is known about development of CLPs and their downstream progeny. Flt3 may be required for the specification of the LMPPs, because Flt3- and Flt3-ligand (L)-deficient mice display a marked decrease in B cell progenitors, their HSCs are impaired in their ability to differentiate into myeloid and lymphoid cells, and recent evidence suggests that Flt3L may influence development of LMPPs (Ceredig et al., 2006; Mackarehtschian et al., 1995; McKenna et al., 2000; Schwarz et al., 2007; Sitnicka et al., 2002).

The emergence of CLPs from LMPPs is demarcated by increased expression of the α chain of the receptor for interleukin 7 (IL-7Rα). The IL-7R is composed of the common γ chain (*Il2rg*) and IL-7Rα (*Il7r*); the latter is also a component of the thymic-stromal-derived lymphopoietin receptor (TSLP-R) (Kang and Der, 2004). Consistent with IL-7Rα being required for two different receptors that function in B cell development, IL-7Rα-deficient mice have a more pronounced B cell deficiency than *Il2rg*$^{-/-}$ mice, which have an intact TSLP-R (Peschon et al., 1994; Vosshenrich et al., 2003). Although IL-7Rα expression marks CLPs, IL-7R signaling is not absolutely required for generation of these cells. IL-7- and IL-7Rα-deficient mice show a 3-fold decrease in CLP numbers but are profoundly impaired in their ability to differentiate into pre-pro-B lymphocytes and to undergo cytokine-induced expansion (Dias et al., 2005; Kikuchi et al., 2005). As described in the next section, this decreased B cell differentiation suggests a role for IL-7R-modulated transcription factors in the network controlling induction of the B cell-lineage-specification factor EBF1 (Dias et al., 2005; Kikuchi et al., 2005). Importantly, mice lacking both Flt3- and IL-7R-derived signals fail to develop any B cells, demonstrating that together these receptors and their ligands are essential for virtually all B cell development (Sitnicka et al., 2003; Vosshenrich et al., 2003).

To date, no transcription factors have been identified that are definitively required for specification of LMPPs from MPPs. The closest candidate is PU.1, a hematopoietic-specific member of the ETS family of transcription factors (Table 1). Mice harboring a germline mutation in *Sfpi1* (the gene encoding PU.1) die during late embryogenesis or shortly after birth and have severely impaired myeloid and lymphoid differentiation (McKercher et al., 1996; Scott et al., 1994). *Sfpi1*$^{-/-}$ embryos lack identifiable B cells in the liver, as well as T cells in the thymus, suggesting a block in development at or before a common progenitor of these cells or distinct roles for PU.1 in both lineages (DeKoter et al., 2002; Scott et al., 1994). Flt3, the definitive marker of LMPPs, is not expressed at wild-type amounts in *Sfpi1*$^{-/-}$ embryos, making it difficult to ascertain whether PU.1 is required for development of LMPPs, or whether PU.1 functions after specification to regulate differentiation from LMPPs, as was recently demonstrated for Ikaros (see below). Nonetheless, PU.1 functions very early in the stepwise progression toward B cell specification.

Previous studies implicated PU.1 dose as a determinant of B lymphocyte versus macrophage specification (DeKoter and Singh, 2000). This conclusion was based on the observation that PU.1-deficient fetal-liver cells transduced with a PU.1-producing retrovirus formed macrophages with high ectopic PU.1 expression, whereas B cells expressed substantially lower amounts of ectopic PU.1 (DeKoter and Singh, 2000). This finding parallels the known differential expression of PU.1 in B cells and macrophages (Nutt et al., 2005). However, this model for PU.1-dose-driven specification has been challenged by the finding that PU.1 expression is similar in HSCs, CLPs, and CMPs when assayed in bone marrow from adult mice carrying an IRES-GFP sequence inserted into the endogenous *Sfpi1* locus (Back et al., 2005; Dakic et al., 2007; Nutt et al., 2005). These data suggest that in adult bone marrow, the dichotomy in PU.1 expression is revealed only after specification to the macrophage or B cell lineages.

Until recently, it was believed that PU.1 was absolutely required for the formation of clonogenic B cell progenitors (pro-B cells), potentially because of its role in regulating *Il7r* expression (DeKoter et al., 2002); however Graf and colleagues demonstrate that *Sfp1*$^{-/-}$ B cell colonies can

be derived from fetal-liver progenitors, albeit at a low frequency and with slower kinetics than from wild-type embryos (Ye et al., 2005). The embryonic lethality that results from PU.1 deficiency has hampered any further understanding of the function of PU.1 in adult bone-marrow lymphopoiesis. Recently this bottleneck has been overcome with both PU.1 conditional mutant mice and hypomorphic mutations that knock down PU.1 expression but still result in viable adult mice (reviewed by Dakic et al. [2007]). The conditional inactivation of PU.1 throughout adult bone marrow results in a greatly perturbed hematopoiesis and, in surprising contrast to fetal-liver development, markedly excess granulopoiesis (Dakic et al., 2005). Adult PU.1-deficient mice lack an identifiable CLP population and are not able to contribute to the lymphoid lineages in a competitive-reconstitution assays, indicating that PU.1 functions at or before the CLP stage. Surprisingly, inactivation of PU.1 in committed B cells with a Cre recombinase driven by the *Cd19* promoter allowed relatively normal B cell differentiation and function (Polli et al., 2005; Ye et al., 2005). Moreover, in vitro inactivation of PU.1 in sorted CLP also allowed B cell differentiation, suggesting that PU.1 functions primarily to specify lymphoid progenitors but is not required for further B lymphocyte differentiation (Iwasaki et al., 2005).

A second genetic model of PU.1 function in adults was produced by the deletion of an upstream regulatory element in the *Sfpi1* gene resulting in a 3-fold reduction in PU.1 expression and a profound block in B cell development at an early stage (Rosenbauer et al., 2006). Recently an alternative hypomorphic allele of *Sfpi1* was described in a study that reached a similar conclusion (Houston et al., 2007). Interestingly, lowering PU.1 expression preferentially allows B1 B cell development (Rosenbauer et al., 2006). A similar expansion of B1 cells was reported in aged mice after conditionally inactivating PU.1 in B lymphocytes, suggesting a role for PU.1 in specifying or maintaining the B1 versus conventional (B2) B cell fate (Ye et al., 2005). Although these experiments clearly point to an essential role for PU.1 in early lymphopoiesis, the factors that regulate PU.1 expression and the molecular targets involved in initial lymphoid specification remain to be determined.

A second transcriptional regulator implicated in early lymphoid specification is Ikaros (Table 1). The Ikaros gene (*Ikzf1*) encodes multiple transcription factors through alternative splicing of exons encoding the zinc fingers involved in DNA binding (Molnar and Georgopoulos, 1994). The Ikaros proteins can function as transcriptional activators or repressors and may function as antagonists of other Ikaros or related transcription factors (Ng et al., 2007). Biochemical studies have shown that in T cell and erythroid progenitors, Ikaros is associated with two chromatin remodeling complexes, the NuRD (nucleosome remodeling and deacetylase) and the SWI-SNF complex (Kim et al., 1999; O'Neill et al., 2000). This finding, along with the accumulation of Ikaros protein at pericentric heterochromatin where it colocalizes with repressed genes, suggests that Ikaros functions predominantly as a transcriptional repressor (Brown et al., 1997). However, the regulation of target genes is likely to be gene specific because Ikaros can both activate and repress reporter plasmids in transfection assays and endogenous genes (Harker et al., 2002; Trinh et al., 2001).

Loss-of-function and dominant-negative experiments demonstrated that Ikaros is essential for multiple aspects of lymphoid development (Georgopoulos et al., 1994; Wang et al., 1996). *Ikzf1*$^{-/-}$ mice completely lack B cells from the earliest detectable stage (Wang et al., 1996), whereas a hypomorphic allele of Ikaros results in impaired ability to undergo the pro- to pre-B cell transition and to form IL-7-dependent pro-B cell colonies in vitro (Kirstetter et al., 2002). Interestingly, postnatal, but not fetal, T cell development proceeds in Ikaros-deficient mice (Wang et al., 1996).

Determining the point at which Ikaros is essential for B lymphopoiesis has proven a difficult challenge. Ikaros-deficient hematopoietic progenitors lack expression of Flt3, leading to the apparent loss of LMPPs; however, expression of an *Ikzf1* promoter/enhancer-driven GFP transgene in these cells revealed that LMPPs develop in Ikaros-deficient mice (Yoshida et al., 2006). In the absence of Ikaros, LMPPs are impaired in B cell and T cell developmental potential and displayed reduced expression of lymphoid genes including *Il7r* and *Rag1*. These data suggest that Ikaros is not essential for development of LMPPs but is required for their further specification into the lymphoid pathway. Mechanistically, this may be because of failed expression of the IL-7R and Flt3, suggesting overlapping functions for Ikaros and PU.1 in early lymphoid specification (Figure 1). Nonetheless, it remains to be determined whether transcriptional-activating or -repressing functions of Ikaros are critical for its lymphoid-specification functions.

The basic helix-loop-helix proteins, E12 and E47, collectively known as E2A, although extensively characterized as regulators of B cell lineage specification, are also required for proper formation of CLPs (Bain et al., 1997; Borghesi et al., 2005). In the absence of E2A, no B cell progenitors develop owing to a requirement for E2A in the induction of EBF1 (see below). E2A regulates expression of many lymphoid genes, but loss of these genes is not predicted to lead to impaired CLP development. Therefore, the mechanism underlying the decreased number of CLPs in these mice remains to be determined.

Specification of the B Lymphocyte Program

One of the critical events in specification of the B cell developmental program is induction of early B cell factor-1 (*Ebf1*). EBF1 is the founding member of the COE (Collier-Olf-EBF) family of transcription factors that bind DNA through an amino-terminal domain containing a novel zinc-coordination motif (Hagman and Lukin, 2005). EBF1 contains a helix-loop-helix (HLH) domain, required for dimerization, and a C-terminal activation domain that is not absolutely required for EBF1 function (Hagman and Lukin, 2005). Mice lacking EBF1 fail to express most B cell genes including *Cd79a* (mb-1, Igα), *Cd79b* (Igβ, B29), *Igll1*

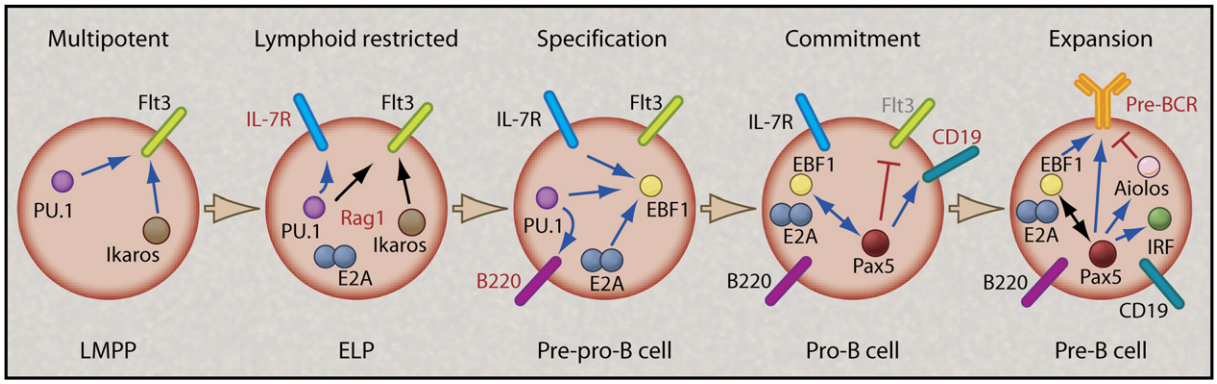

Figure 1. Multistep Model of B Cell Development
Successive stages of differentiation from the LMPP (lymphoid-primed multipotent progenitor), ELP (early lymphoid progenitor), pre-pro-B cell, and committed pro- and pre-B cell are depicted. Developmental capacities of the successive stages are indicated. Key transcription factors, growth-factor receptors, and cell-surface markers are shown, with important events initiated at a particular stage shown in blue. An arrow pointing upward indicates positive interactions, and ⊥ indicates gene repression. RAG1 expression is initiated in the ELP and is maintained until throughout the remaining stages depicted. IRFs, interferon regulatory factor-4 and -8; and preBCR, pre-B cell receptor.

(λ5), and *VpreB1* and do not undergo any *Igh* recombination in the bone marrow (Lin and Grosschedl, 1995). Retrovirus-mediated expression of EBF1 in HSCs skews differentiation toward the B cell lineage, suggesting that, in the context of other lymphoid-specific transcription factors, EBF1 appears sufficient to activate the B cell-lineage gene program (Zhang et al., 2003). This hypothesis gained further support when it was demonstrated that ectopic expression of EBF1 is able to rescue B lymphocyte differentiation from multipotent progenitors blocked at earlier stages of development because of targeted deletion of key lymphoid transcription factors (Table 1). Ectopic expression of EBF1 in E2A-deficient (*Tcfe2a*$^{-/-}$) HSCs rescues B lymphocyte differentiation in vitro (Bain et al., 1994; Seet et al., 2004). However, *Tcfe2a*$^{-/-}$ EBF1-expressing pro-B cells fail to proliferate in response to IL-7 because of a requirement for E2A in IL-7R-induced N-myc (*mycn*) upregulation (Seet et al., 2004). EBF1 also rescues B lymphocyte differentiation, but not IL7-dependent expansion, from *Il7r*$^{-/-}$ pre-pro-B cells or *IL7*$^{-/-}$ CLPs, which fail to express *Ebf1* (Dias et al., 2005; Kikuchi et al., 2005). In addition, B cells develop in vitro from *Sfpi1*$^{-/-}$ HSCs forced to express EBF1 (Medina et al., 2004). However, because a small number of B lymphocytes develop from *Sfpi1*$^{-/-}$ fetal-liver HSCs cultured in vitro, PU.1 may facilitate, but not be essential for, *Ebf1* expression (Ye et al., 2005). In each of these cases, ectopic expression of Pax5, a paired-domain transcription factor essential for B cell lineage commitment (see next section), did not rescue B lymphopoiesis, indicating that EBF1 performs other functions in addition to activating *Pax5* expression (Dias et al., 2005; Kikuchi et al., 2005; Medina et al., 2004; Seet et al., 2004). Taken together, these studies indicate that EBF1 is an essential specification factor for the B cell lineage.

The central role of EBF1 in B cell lineage specification brings to the forefront the question of how *Ebf1* is regulated. Determining the combinatorial inputs to this gene is likely to reveal the factors and mechanisms underlying activation of the B cell program. One of the first regulators of *Ebf1* to be identified was E2A. E2A induces *Ebf1* in a macrophage line, and together these proteins cooperatively regulate most B cell-specific genes, resulting in conversion of these cells to the B cell lineage (Kee and Murre, 1998; Sigvardsson et al., 1997). E box sequences, the DNA target for E2A, are present in the *Ebf1* promoter, indicating that *Ebf1* is a bona fide E2A target gene (see Smith et al. [2002] and Figure 2A). Surprisingly, however, inhibition of E protein activity (E2A is one of three E box-binding proteins, E proteins) in pro-B lymphocytes in vitro, or deletion of E2A in pre-B cell lines, had less of an impact on *Ebf1* mRNA than was predicted by these previous studies (Kee, 2005; Lazorchak et al., 2006). The recent characterization of two *Ebf1* promoters has helped to resolve this conundrum (Roessler et al., 2007).

Ebf1 is controlled through two promoters, a distal promoter (α) and a proximal promoter (β) that produce two EBF1 proteins that differ by 11 amino acids in the N terminus (see Roessler et al. [2007] and Figure 2A). In transient-transfection assays, these distinct proteins activate the *Igll1* promoter equivalently, indicating that the alternative proteins are probably a consequence of the need for differential promoter regulation rather than distinct biological functions. The *Ebf1α* promoter is active in a pro-B lymphocyte line (PD31) and transient-transfection assays reveal regulation by E2A and indirectly STAT5 (Roessler et al., 2007). Because STAT5 is activated by IL-7R signaling, this finding provides a possible explanation for the dependence of initial *Ebf1* expression on both E2A and IL-7R (Dias et al., 2005; Kikuchi et al., 2005; Seet et al., 2004) (Figure 1). EBF-binding sites are also present in the *Ebf1α* promoter, suggesting an autoregulatory function for EBF1 (Roessler et al., 2007). The *Ebf1β* promoter shows preferential activity in a mature B lymphocyte line (Raji) and is regulated by Ets1, PU.1, and Pax5 (Roessler et al., 2007). A role for Pax5 in promoting *Ebf1* expression was also

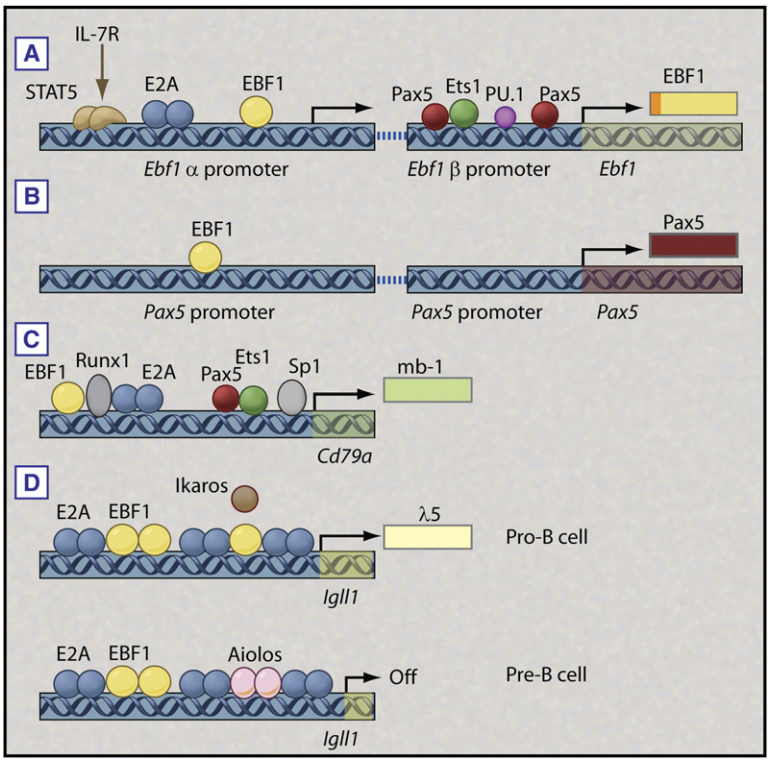

Figure 2. Combinatorial Control of B Cell-Specific Gene Expression

(A) Regulation of the *Ebf1* gene. The two promoters of *Ebf1* along with the known regulators are indicated. The EBF1 protein is shown in yellow. The gene product of the two promoters differs by 11 amino acids in the N terminus (shown in orange). The IL-7R-STAT5 pathway has been shown to influence *Ebf1*α promoter activity, although direct DNA binding has not been demonstrated. Although Pax5 binds to the *Ebf1*β promoter, the functionality of individual Pax5-binding sites for *Ebf1* expression has not been confirmed.

(B) Regulation of the *Pax5* gene. *Pax5*, like *Ebf1*, has two independent promoters; however, beyond a role for EBF1, very little is known about the control of *Pax5* transcription.

(C) *Cd79a* is regulated by the combinatorial inputs from E2A, EBF1, Pax5, Ets1, Sp1, and Runx1. Ets1 can only bind to a nonclassical site via cooperative interaction with Pax5 in the absence of DNA methylation (a process that requires E2A, EBF1, and Runx1 binding).

(D) The *Igll1* promoter is regulated by competition between Ikaros family members (Ikaros and Aiolos) and EBF1 for overlapping binding sites. In pro-B cells, relatively higher levels of EBF1 favor *Igll1* activation. However, at the pre-B cell stage, pre-BCR signaling leads to increased expression of Aiolos, which promotes gene silencing.

suggested by the finding that ectopic expression of Pax5 in T cell progenitors induced *Ebf1* (Fuxa et al., 2004). Because expression of *Pax5* is dependent on EBF1, EBF1 regulates its own expression directly through induction of the *Ebf1*α promoter and indirectly through upregulation of *Pax5*. Compared to wild-type pro-B cells, *Pax5*$^{-/-}$ pro-B cells have reduced *Ebf1* mRNA that is predominantly because of decreased transcription from the *Ebf1*β promoter (Roessler et al., 2007). This feedback regulation by Pax5 on *Ebf1* could function to amplify B cell-specific gene expression and solidify commitment to the B cell pathway (Figure 1). These studies suggest a progressive *Ebf1* induction occurring through three phases as follows: (1) E2A- and STAT5-dependent activation of *Ebf1*α; this is followed by (2) EBF1 enhanced expression from the *Ebf1*α promoter and induction of *Pax5* and, finally, (3) Pax5-, Ets1-, and PU.1-directed activation of the *Ebf1*β promoter (Figure 2A). Nonetheless, in all primary lymphoid populations expressing *Ebf1*, including CLPs, transcripts initiating from both *Ebf1*α and *Ebf1*β are detected and the *Ebf1*β-derived transcripts are most prevalent (Roessler et al., 2007). Therefore, these three phases of *Ebf1* induction may occur in rapid succession and may not be truly separable by the developmental stage.

Major insights into the combinatorial mechanisms promoting lineage-specific gene expression have come from analysis of the regulatory regions of B cell-specific genes. Although our knowledge is far from complete, studies of the immunoglobulin heavy (*Igh*) and light chains (*Igk* and *Igl*), *Igll1-VpreB1*, *Rag1-Rag2*, and *Cd79a* led to the identification of numerous transcription factors, their mechanisms of DNA binding, and transcription activation either alone or in cooperation with other factors (Hagman and Lukin, 2005; Hsu et al., 2003; Sabbattini and Dillon, 2005; Schlissel, 2004). These studies revealed that *cis*-acting regulatory regions in many B cell genes are cooperatively activated by, at a minimum, E2A and EBF1. Consistent with this finding, mice with compound heterozygous mutations in *Tcfe2a* and *Ebf1* fail to express many B cell genes in the bone marrow and B lymphopoiesis is arrested at an early pro-B cell stage (O'Riordan and Grosschedl, 1999). However, in the presence of ectopic EBF1, the functions of E2A can be partially replaced by the low amount of related E proteins expressed in B lineage cells (Seet et al., 2004; Zhuang et al., 1996). Although many transcription factors cooperate to regulate B cell genes, to date, E2A and EBF1 are the only factors whose synergistic activity has been demonstrated to be required for B lymphopoiesis. The requirement for cooperation between these factors may be due to regulation of the essential B lineage-commitment factor Pax5. However, surprisingly little is known about the mechanisms controlling expression of *Pax5*; only EBF1 and Stat5 have been directly examined as regulators of this gene (Figure 2B) (Hirokawa et al., 2003; O'Riordan and Grosschedl, 1999).

The *Cd79a* and *Igll1* genes have been useful models for analysis of *cis*- and *trans*-regulatory mechanisms controlling B cell gene expression (Figures 2C and 2D). *Cd79a* encodes a transmembrane ITAM (immunoreceptor tyrosine-based activation motif)-containing protein essential for pre-B cell receptor (BCR) and mature BCR signal transduction (Clark et al., 2005). The *Cd79a* promoter proximal

enhancer is regulated by combinatorial inputs from E2A, EBF1, Pax5, Ets1, and Runx1 (also known as AML1) (Hagman and Lukin, 2005). Early analysis of the *Cd79a* enhancer led to the identification, purification, and cloning of EBF1 (Hagman et al., 1993). Subsequent studies revealed a nonclassical Ets-binding site that can be bound by Ets1 only through cooperative interaction with Pax5, which induces a conformational change that allows DNA binding in Ets1 (Fitzsimmons et al., 1996). However, even in the presence of Pax5, binding of Ets1 to the *Cd79a* enhancer requires demethylated DNA, which is achieved only after binding of E2A, EBF1, and Runx1 to sequences upstream of the composite Pax5 and Ets site (Maier et al., 2004).

The mechanisms controlling expression of *Igll1* (λ5) have been studied extensively both because it is a B cell-restricted gene and because its expression is extinguished after successful rearrangement of the *Igh* gene, during transition to the pre-B cell stage (Martensson et al., 2001). B cell-specific expression of *Igll1* is ensured by sequestration of the *Igll1* gene in centromeric heterochromatin by repressive complexes containing Ikaros (Brown et al., 1997). Interestingly, a recent study demonstrated that Ikaros-family proteins compete with EBF1 for binding to the *Igll1* promoter, because both factors share overlapping binding sites (Thompson et al., 2007) (Figure 2D). During induction of the B cell gene program, increasing amounts of EBF1 are likely to tip the balance toward *Igll1* induction, given that other essential regulators, such as E2A, are present (Sigvardsson et al., 1997). Subsequent downregulation of the *Igll1* gene in pre-B cells requires Aiolos, an Ikaros-related protein, that is induced after pre-BCR signaling (Thompson et al., 2007) (Table 1). Therefore, repression of *Igll1* involves not only expression of repressive DNA-binding factors but also efficient competition with positive regulators such as EBF1.

B Cell Commitment

For many years, lineage specification and commitment were considered as a single synonymous event; however, the finding that transcripts from multiple lineages could be found in multipotent cells, a phenomenon referred to as lineage priming, suggested that the specification and commitment processes were temporally and genetically distinct (Hu et al., 1997; Traver and Akashi, 2004). The B cell lineage provides one of the best examples of the dissociation of these processes, with factors such as EBF1 and E2A functioning directly in B cell specification and indirectly controlling commitment through induction of *Pax5*, which is required to complete the commitment process (Table 1).

Pax5 is a multifunctional transcriptional regulator that is expressed at a remarkably stable amount throughout the B cell lineage, from the pro-B cell stage until its downregulation in plasma cells (Fuxa and Busslinger, 2007). Pax5 binds to DNA through an N-terminal paired-domain motif and can both positively and negatively regulate transcription (reviewed by Cobaleda et al. [2007]). In the absence of Pax5, B cell development is arrested at the early pro-B cell (or pre-BI) stage of differentiation characterized by expression of many B cell-specific transcripts and D-J_H rearrangements at the *Igh* locus (Nutt et al., 1997). Intriguingly, although *Pax5*$^{-/-}$ pro-B cells are unable to differentiate into mature B cells, they can be cultivated indefinitely in vitro in the presence of IL-7 and stroma. Most surprising, however, is that these pro-B cells are not committed to the B cell lineage but instead are capable of differentiating into a broad spectrum of hematopoietic cell types (Nutt et al., 1999; Rolink et al., 1999). Restoration of Pax5 expression in *Pax5*$^{-/-}$ pro-B cells suppresses this multilineage potential, whereas conditional inactivation of *Pax5* in pro-B cells reverts lineage commitment and allows reacquisition of multilineage potential (Mikkola et al., 2002). A similar capacity for multilineage differentiation was reported for E2A-deficient lymphoid cell lines, and such a finding is in keeping with the fact that these cells lack high expression of markers of B cell specification as well as *Pax5* expression (Ikawa et al., 2004).

The requirement for Pax5 in both initiating and maintaining B cell commitment has generated intense interest in understanding the mechanisms by which Pax5 controls gene expression and the nature of the target genes. The ability to propagate the *Pax5*$^{-/-}$ pro-B cell lines has been a crucial asset for the analysis of Pax5-dependent gene expression (Nutt et al., 1998). Because Pax5 has the ability to both activate and repress genes, it was hypothesized that Pax5 may promote B cell commitment by repressing the expression of non-B cell genes. In keeping with this concept, *Pax5*$^{-/-}$ pro-B cells express many genes associated with multipotent progenitors or non-B lineage cells (Delogu et al., 2006; Nutt et al., 1999). Striking examples include the genes encoding the cell-surface receptors MCSF-R and Notch1, associated with macrophage and T cell development, respectively, and their expression provides a molecular explanation for the lineage plasticity of the *Pax5*$^{-/-}$ pro-B cells (Souabni et al., 2002; Tagoh et al., 2006). Another key target of Pax5-mediated repression is *Flt3*, which is directly repressed by Pax5 upon lineage commitment (Delogu et al., 2006; Holmes et al., 2006) (Figure 1). This repression is crucial for B lymphopoiesis because enforced expression of Flt3 throughout hematopoiesis or injection of saturating amounts of Flt3L blocks B cell formation potentially by diverting progenitors down the DC pathway (Ceredig et al., 2006; Holmes et al., 2006). Therefore, Pax5 functions to promote B cell lineage commitment through repression of essential receptors for other differentiation pathways.

Global transcriptional profiling has been employed with great success to identify Pax5-repressed genes (Delogu et al., 2006). Using this approach, >100 Pax5-repressed genes that are involved in many biological processes have been identified and validated, with the majority of these target genes normally expressed in non-B cell lineages. Surprisingly, the conditional inactivation of Pax5 in committed pro-B cells or mature B cells resulted in the reactivation of many of these repressed genes; such a reactivation also occurs to some degree after the physiological downregulation of Pax5 during plasma-cell differentiation

(Delogu et al., 2006; Kallies et al., 2007; Nera et al., 2006). This finding is consistent with the reacquisition of multilineage differentiation after inactivation of Pax5 and confirms that B cell lineage commitment needs to be continually maintained by Pax5.

With a candidate-gene approach, a number of positively regulated Pax5 targets have been identified, including many components of the pre-BCR and BCR and associated signaling molecules such as CD19, Blnk, CD79a, and λ5 (see Cobaleda et al. [2007] and Figure 1). As described in the preceding section, *Cd79a* is a well-characterized Pax5-regulated gene and exemplifies the combinatorial nature of B cell-specific gene expression.

Very recent gene-profiling data indicate that Pax5 activates a similar number of genes in B cells as it represses (A. Schebesta and M. Busslinger, personal communication, and S.L.N., unpublished data). The list of activated genes includes genes that encode a number of transcription factors important for various aspects of B cell differentiation, including SpiB, Aiolos, Id3, Lef1, IRF4, and IRF8, suggesting that Pax5 activity initiates a cascade that acts to reinforce B cell commitment and subsequent B cell differentiation (Figure 1). Interestingly, the genes for two factors E2A and EBF1 that, as outlined above, are crucial for B cell specification and thought to act upstream of Pax5 are also upregulated by Pax5 (see Roessler et al. [2007] and S.L.N., unpublished data). These data support the model derived from the molecular dissection of *Ebf1* gene regulation and demonstrate that the transcriptional network controlling B cell specification and commitment is not a simple linear cascade but involves multiple combinatorial inputs and feedback loops.

Orphan B Cell Transcription Factors

A number of transcription factors are known to be essential for development of pro-B lymphocytes, but their precise functions and essential target genes have not been determined (Table 1). Moreover, how these factors fit into the network of regulatory factors controlling B lymphopoiesis remains to be clarified. Lymphoid enhancer factor 1 (Lef1) is a high-mobility group (HMG)-box protein that activates transcription in response to Wnt signaling, through interactions with β-catenin, but it can also function as a repressor or architectural protein in the absence of Wnt signaling (Staal and Clevers, 2005). Embryonic and neonatal mice lacking Lef1 have a 2-fold reduction in the number of pro-B cells, and in vivo these cells proliferate less well than wild-type pro-B cells (Reya et al., 2000). In vitro, *Lef1*$^{-/-}$ pro-B lymphocytes proliferate in response to IL-7, but not Wnt3a, indicating that Wnt signaling may be required for expansion of pro-B lymphocytes; however, the essential targets of Lef1 in fetal-liver pro-B cells have not been determined. Sox4 is another HMG-box transcription factor that is essential for B cell development. The Sox4 deficiency results in embryonic lethality, and E13.5 fetal-liver cells from *Sox4*$^{-/-}$ embryos fail to expand in IL-7-supplemented in vitro culture and produce few B lymphocyte progenitors after transfer into irradiated adult recipients (Schilham et al., 1996). Sox4 interacts with the PDZ-domain protein syntenin, a protein associated with signaling through IL-5Rα, suggesting that Sox4 might function directly in membrane-proximal signaling pathways (Geijsen et al., 2001). Nonetheless, the signaling pathways in pro-B lymphocytes leading to activation of Sox4 and essential Sox4 target genes remain to be identified.

The zinc-finger transcription repressor Bcl11a (*Evi9*) has also been implicated in early B lymphopoiesis, and mutations leading to activation of this gene are found in B cell lymphoma (Liu et al., 2003; Satterwhite et al., 2001). Bcl11a is also required for proper T lymphopoiesis; however, it remains to be determined whether Bcl11a is required in multipotent progenitors or specifically in cells committed to B and T cell differentiation. Another factor that has recently been implicated in the early stages of B cell development is the GA-binding protein (GABP). GABP is a ubiquitously expressed Ets-family transcription factor that consists of α and β subunits, which mediate DNA binding and transcriptional activation, respectively (Sharrocks, 2001). GABP regulates the expression of the IL-7R in T cells, whereas it is found to be dispensable for IL-7R expression in B cells (Xue et al., 2004). The recent analysis of mice homozygous for a gene-trapped *gabpa* allele that results in hypomorphic expression shows impaired B cell development characterized by a very low frequency of clonogenic pro-B cells and a block in differentiation at the pro-B cell stage (Xue et al., 2007). Gene-expression analysis revealed decreased expression of a number of B cell-specific genes in *gabpa* mutants, suggesting a mechanism by which GABP regulates B cell development.

Conclusions

The detailed analysis of the small number of key transcription factors that coordinate B cell specification and commitment has revealed that the transcriptional control of early B cell development does not proceed in a linear fashion. Rather, this process involves hierarchical forward steps and feedback loops, with this handful of factors being used in multiple contexts and distinct combinations. It has also demonstrated the surprising requirement for continual reinforcement of the commitment process throughout the life of a B cell. Although these studies have facilitated the development of the cellular and molecular model of B cell lineage specification and commitment outlined here, many questions remain. For example, our knowledge of the crucial target genes of PU.1, Ikaros, and E2A required for promoting lymphoid specification remains limited. In addition, despite being crucial to the commitment process, little is known about the factors that regulate *Pax5* in B cells. Finally, the "orphan" B cell factors, such as Sox4, need to be incorporated into the model. Although resolving these questions will be essential for an understanding of the B cell-commitment process, they also have important practical consequences as revealed by the recent identification of most of these transcriptional regulators as targets of mutation or deletion in mouse and human acute lymphoblastic leukemia (see Mullighan et al. [2007] and Table 1). These findings demonstrate that the function of these essential B cell

transcription factors needs to be carefully controlled to avoid unwanted outcomes such as malignancy.

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