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The regulatory network of B-cell differentiation: a focused view of early B-cell factor 1 function

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Summary: During the last decades, many studies have investigated the transcriptional and epigenetic regulation of lineage decision in the hematopoietic system. These efforts led to a model in which extrinsic signals and intrinsic cues establish a permissive chromatin context upon which a regulatory network of transcription factors and epigenetic modifiers act to guide the differentiation of hematopoietic lineages. These networks include lineage-specific factors that further modify the epigenetic landscape and promote the generation of specific cell types. The process of B lymphopoiesis requires a set of transcription factors, including Ikaros, PU.1, E2A, and FoxO1 to 'prime' *cis*-regulatory regions for subsequent activation by the B-lineage-specific transcription factors EBF1 and Pax-5. The expression of EBF1 is initiated by the combined action of E2A and FoxO1, and it is further enhanced and maintained by several positive feedback loops that include Pax-5 and IL-7 signaling. EBF1 acts in concert with Ikaros, PU.1, Runx1, E2A, FoxO1, and Pax-5 to establish the B cell-specific transcription profile. EBF1 and Pax-5 also collaborate to repress alternative cell fates and lock cells into the B-lineage fate. In addition to the functions of EBF1 in establishing and maintaining B-cell identity, EBF1 is required to coordinate differentiation with cell proliferation and survival.

Keywords: *EBF1, B-cell differentiation, regulatory network, lineage specification, B-cell commitment*

Introduction

Hematopoiesis is one of the best characterized developmental systems for studying cell fate decisions, differentiation, lineage-specific gene expression, as well as the stability and plasticity of cellular phenotypes. In particular, B lymphopoiesis is an excellent paradigm for the stepwise differentiation of a multipotent progenitor (MPP) into a terminally differentiated effector cell. The differentiation process can be monitored by the expression of surface antigens, the rearrangement status of the heavy and light chain of the immunoglobulin genes and by the expression of certain genes like *Rag1* and *Rag2* (1). Differentiation of a hematopoietic stem cell (HSC) into highly specialized antibody-producing B cells involves the acquisition of cell type-specific gene expression signatures (specification) and the loss of the ability to

differentiate into alternative cell lineages (commitment) (2–4). Specialized stromal cells guide the developmental progress by providing cell–cell interactions and secreting cytokines and chemokines (5). The microenvironment influences not only the survival and proliferation of cells but also affects their responsiveness to external signals, thereby shaping the transcriptional network of the developing cell. A set of transcription factors, including Ikaros, Runx1/Cbfβ, E2A (Tcf3), and FoxO1, directs cells into the B-cell lineage by providing an epigenetic landscape that is permissive for the action of B cell-specific factors, including early B-cell factor 1 (EBF1) and paired box transcription factor 5 (Pax-5). Together with other transcription factors, EBF1 and Pax-5 activate the transcriptional program that eventually leads to the generation of mature B cells, and they repress alternative lineage choices (6–9). During the last few years, it has become clear that the regulatory system underlying B-cell differentiation does not involve a simple linear hierarchy in which transcription factors are sequentially activated. Instead, B lymphopoiesis requires a complex regulatory network in which transcription factors are interconnected via feed-forward and feedback loops and cross-antagonism. Moreover, their expression or activity can be further modulated by signaling pathways and epigenetic regulation. In this review, we present the current view of the regulatory network governing B-cell differentiation with a focus on one key determinant, EBF1.

B-cell lymphopoiesis

B-cell differentiation starts in the fetal liver or adult bone marrow with an asymmetric cell division of a HSC and the generation of a MPP (10) (*Fig.* 1). This progenitor acts as a branching point for the myeloid and lymphoid lineages (11). Differentiation along the myeloid lineage is initiated by the common myeloid progenitor (CMP) that generates megakaryocytes, erythrocytes, granulocytes, and macrophages (12). The lymphoid lineage is marked by the surface expression of the tyrosine kinase Flt3 receptor that is first detected on the lymphoid-primed multipotent progenitor (LMPP). Although LMPPs have lost the megakaryocyte–erythroid lineage potential, they can give rise to granulocytes, macrophages, and lymphocytes (13, 14). Reduced expression of the stem cell markers SCA-1 and c-Kit and upregulation of IL-7 receptor expression mark the next step in lymphopoiesis, represented by the common lymphoid progenitor (CLP) (15, 16). Recently, the transmembrane protein Ly6D has been identified as an early B-cell marker that allows the subdivision of CLPs into Ly6D-negative all lymphoid progenitors (ALP) and B-cell-biased lymphoid progenitors (BLP) that express Ly6D on the surface (17). ALPs retain the potential for generating natural killer cells (NK), dendritic cells (DC), T and B cells, whereas BLPs show a markedly reduced T-cell potential and generate predominantly B cells (17). Pre-pro-B cells, also called fraction (Fr.) A in the nomenclature of Hardy and Hayakawa (1), are marked by the B220 isoform of the CD45 receptor but lack canonical B-cell markers including CD19 (18). In the following pro-B-cell stages (Fr.B and Fr.C), the immunoglobulin heavy chain (IgH) genes are rearranged in a Rag-dependent manner. Successful rearrangement culminates in the pairing of heavy chain and surrogate light chains λ5 (*Igll1*) and VpreB1 (*Vpreb1*). Together with its signaling components, Igα (*Cd79a*) and Igβ (*Cd79b*), the pre-B-cell receptor (pre-BCR) is expressed on the surface of large pre-B-cells (Fr. C'). Passing this developmental checkpoint activates rearrangement of the immunoglobulin light genes in the small pre-B-cell stage (Fr.D). Productive recombination of the Igκ or Igλ locus results in the expression of the IgM B-cells receptor (BCR) on the surface of the immature B cell (19). Only a subset of these immature B cells leave the bone marrow and only a portion of them complete their development in the spleen and join the mature B-cell pool (20). Mature B cells circulate in blood and secondary lymphatic organs. After contact with a pathogen-derived antigen, mature B cells undergo class switch recombination (CSR) and somatic hypermutation (SHM) and differentiate into plasma cells that produce high affinity soluble antibodies (21).

Early B-cell factor 1: protein structure and mechanism of action

Protein structure of EBF1

EBF1 is one of the key factors of B-cell differentiation. EBF1 was discovered as a factor with B lineage-specific DNA-binding activity to the *Cd79a* promoter (22). Because of its strong expression in early B cells, the factor was named EBF (22, 23) which was later changed to EBF1. Purification of this factor from a transformed pre-B-cell line by sequence-specific DNA affinity chromatography characterized EBF1 as a dimer of two 65 kDa subunits that binds its palindromic DNA-binding motif 5′-TCCCNNGGGA with high affinity (24). Amino acid sequence analysis allowed for the molecular cloning of EBF1, which was also independently cloned as Olf1 in a yeast-one-hybrid screen, using the 5′ flanking region of the gene encoding olfactory marker protein (*Omp*)

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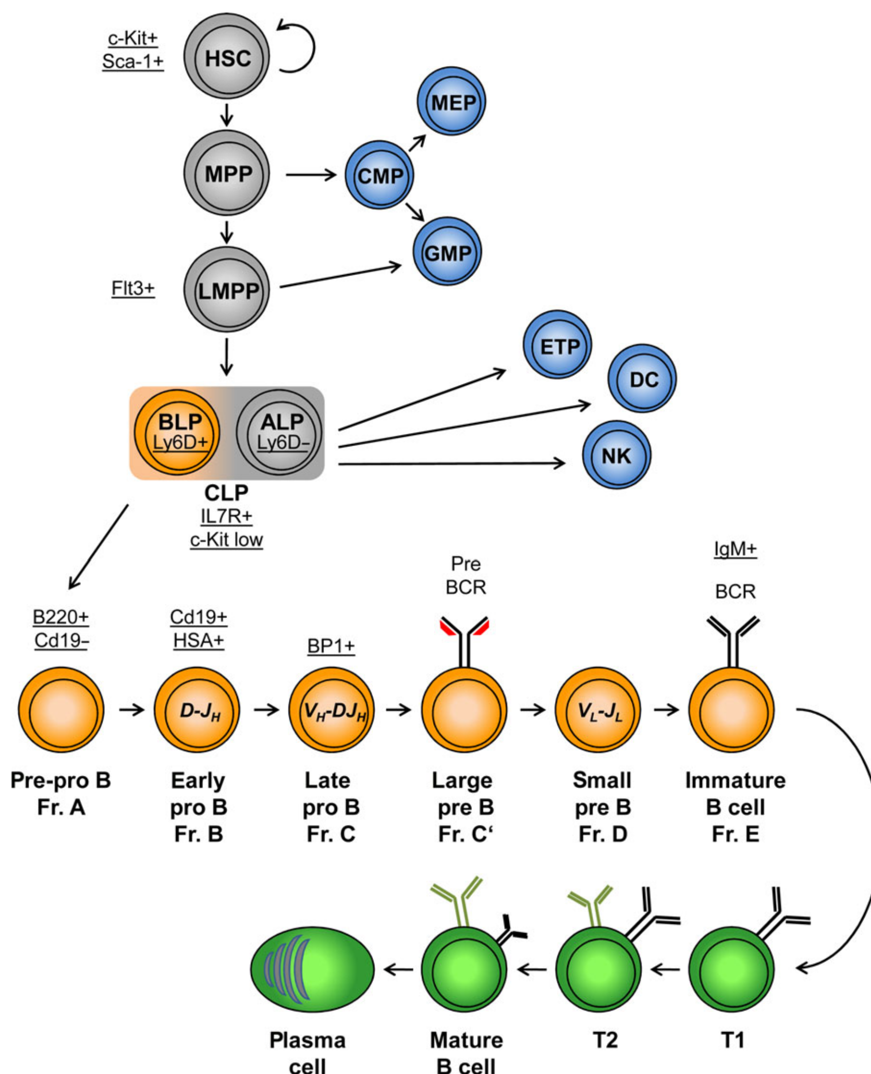

Fig. 1. A schematic view of B-cell lymphopoiesis. Common developmental steps of B and non-B cells are colored in gray. Early B-cell development in the bone marrow is shown in orange, while late B-cell development in the periphery is depicted in green. Non-B cells are colored in blue. The developmental stages are marked by bold letters and the presence or absence of surface proteins, indicative of specific cell types, are underlined. Rearrangements of the heavy and light chains are written in italic letters. HSC, hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; ALP, all lymphoid progenitor; BLP, B-cell-biased lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocytic/erythrocyte progenitor; GMP, ganulocyte/macrophage progenitor; ETP, early thymic progenitor; DC, dendritic cell; NK, natural killer cell; T1 and T2, transitional B cell 1 and 2. Adapted from Mandel and Grosschedl (2), Lai and Kondo (14), Roessler and Grosschedl (19), and Rolink, Andersson, and Melchers (20).

(23, 25, 26). Together with Collier, an Olf-1/EBF ortholog identified in *Drosophila melanogaster*, Olf-1 and EBF1 established a new family of transcription factors, which was named COE according to its founding members. EBF1 is highly conserved during metazoan evolution and shows strong sequence overlap with the three other members of the family, now termed EBF2, EBF3, and EBF4 (27). All COE factors consist of four protein domains: an N-terminal DNA-binding domain (DBD), an IPT (Ig-like/plexins/transcription factors) domain, a helix-loop-helix (HLH) dimerization domain, and a C-terminal transactivation domain.

The N-terminal DNA-binding domain, spanning some 220 amino acids, shows the highest degree of sequence conservation, as the similarity between the evolutionarily most distantly related proteins still exceeds 80% (28, 29). Biochemical analysis of the DBD demonstrated that its interaction with DNA is dependent on a zinc-coordination motif, H-X_3-C-X_2-C-X_5-C, located between amino acids 157 and 170 (29, 30). Because of its difference to the canonical zinc finger structure, this atypical zinc finger motif was termed 'zinc knuckle' or 'COE motif' (29). Methylation interference assays showed that EBF1 contacts both the major and minor

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grooves of DNA (22). Recent determination of the crystal structures of EBF1 and an EBF1:DNA complex clarified the three-dimensional architecture of the DBD and elucidated the interaction between EBF and DNA at atomic resolution (31, 32) (*Fig.* 2). The DBD folds into a β-sandwich, which consists of a four- and a five-stranded anti-parallel β-sheet. DNA interaction is mediated by three distinct DNA interaction modules, the GH loop, a central module, and the zinc knuckle, that extend from the β-sandwich (31). Most of the sequence specificity of DNA binding by EBF1 is provided by the central module. It is composed of small β-sheets and loops that reach deep into the major groove, contacting specific nucleotides of one half-site of the palindromic binding sequence. The zinc knuckle, which consists of short α-helices, contacts the other half-site in the minor groove. The large GH loop protrudes into the minor groove outside of the binding motif. Although the GH loop is important for DNA-binding affinity, it does not appear to contribute to the specificity of sequence recognition (31). The crystal structure confirmed the binding of DNA by a dimer of EBF1 (22). Interestingly, the two monomers of EBF1 form a symmetric clamp over the entire binding motif. In this structure, each monomer contacts both half-sites of the palindromic consensus sequence. Although EBF1 shows no amino acid sequence similarity with other families of DNA-binding proteins, the three-dimensional architecture of the EBF1 DBD resembles the N-terminal half of the Rel-homology domain (RHD), which is found in NF-κB and NFAT (31, 32).

The DBD is followed by an IPT domain that extends from aa 262 to 345 (33). The RRARR motif located between the DBD and the IPT domain was proposed as a putative nuclear localization signal (NLS) (25). As predicted by sequence comparison and underlined by the crystal structure, the IPT domain adopts an immunoglobulin-like fold. It resembles the C-terminal half of the RHD. The structural similarities of

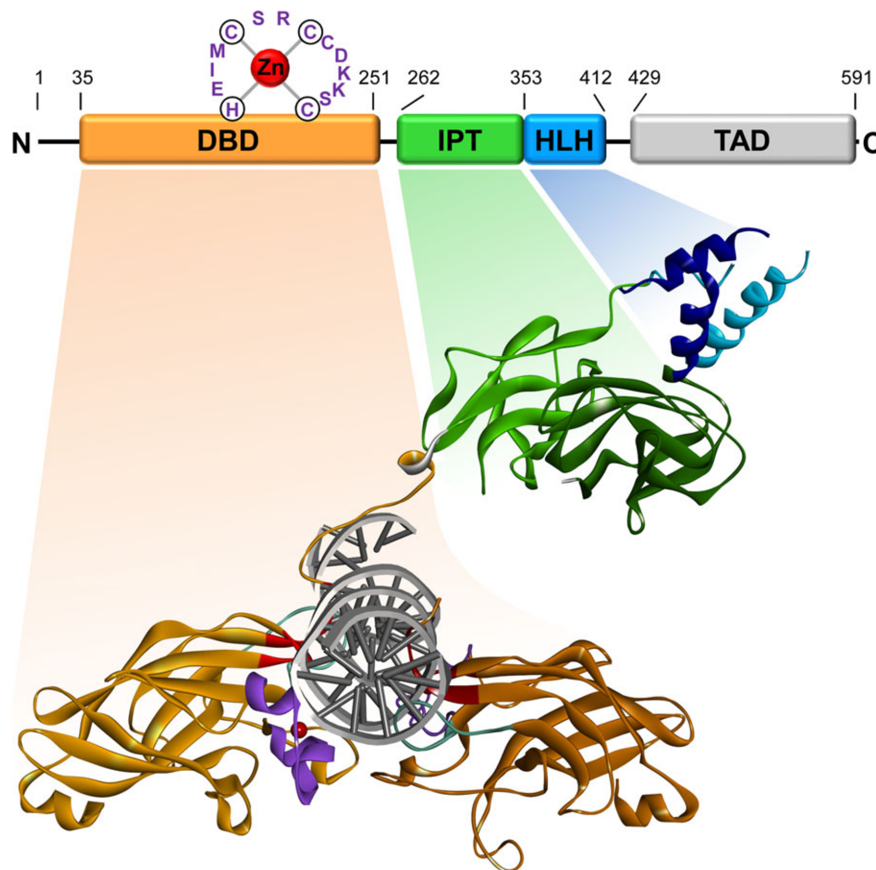

Fig. 2. Structure of EBF1. A schematic presentation of the domain structure of murine EBF1 [modified after Hagman and Lukin (38)] and the crystal structure of a DNA-bound EBF1 dimer that lacks the C-terminal transactivation domain [modified after Treiber *et al.* (31) using PDB file 3MLP are depicted]. The structure was modeled using Discovery Studio 3.5 Visualizer, Accelrys Inc., San Diego, CA. The DNA-binding domain (DBD) is colored in orange, the IPT domain in green, the helix-loop-helix (HLH) dimerization domain in blue and the C-terminal transactivation domain (TAD) in light gray. The DNA interacting modules within the DBD are colored in red (central motif), purple (zinc coordinating motif) and turquoise (GH loop). Zinc ions are represented by red spheres. The border amino acids of each domain are indicated in the schematic.

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both DBD and IPT domain with the RHD strengthen the relationship between EBF1 and members of the Rel family (31, 32). In contrast to NFAT and NF-κB, in which the IPT domain is involved in DNA binding, dimerization and protein–protein interaction (34, 35), the function of the IPT domain of EBF, which is dispensable for DNA binding and dimerization (23), remains elusive.

EBF1 forms stable homo- and heterodimers via an HLH domain, consisting of two amphipathic helices (23, 36, 37). Dimerization of the four helices, two from each monomer, forms a helix bundle comparable to the dimerized basic HLH domains of other proteins like MyoD (31, 38, 39). The second helix is duplicated in vertebrates, resulting in a helix-loop-helix-loop-helix motif. However, the third helix is not essential for dimerization (37). Moreover, the crystal structure of EBF1 argues against an inclusion of the third helix in the HLH dimerization motif and raises the possibility that the third helix-like motif interacts with other proteins (31, 32).

The C-terminal transactivation domain is only poorly conserved between the COE family members. Nevertheless, common properties are a strong enrichment of proline, serine, and threonine residues and a lack of predicted secondary structure. Despite the poor sequence conservation, the C-terminus of all four EBF members in mice contributes to the activation of gene expression in vector-based reporter assays (23, 36, 37). However, EBF4 shows a lower transactivation potential compared to the other family members (37). Aside from the C-terminal domain, an additional transactivating region may be located in the DBD because a truncated version of EBF lacking the C-terminus is still able to activate transcription (29).

The molecular mechanism of EBF1 function

EBF1 is the only COE factor that is expressed in the hematopoietic system, and it plays a critical role in B-cell development (2, 6–8). Genome-wide chromatin immunoprecipitation analysis combined with deep sequencing (ChIP-seq) in pro-B cells identified approximately 5000 EBF1-occupied sites corresponding to some 3000 genes (40, 41). These genes are strongly associated with B-cell function and encode many components of the B-cell receptor signal transduction cascade. However, EBF1 gain-of-function experiments in pre-pro-B cells and EBF1 loss-of-function studies in pro-B cells indicated that only a small fraction of EBF1-bound genes are regulated by EBF1 (40). These studies also revealed that EBF1 can both activate and repress genes. Beside the presence of activated and repressed genes among EBF1-occupied targets, a third group of genes, termed ‘poised’ genes, are bound by EBF1 in early B-cell stages but expression is detected only at later stages of the B lineage (40). Notably, analysis of histone marks linked EBF1 binding to di-methylation of histone 3 at lysine 4 (H3K4me2). Ectopic expression of EBF1 in a pre-T-cell line resulted in H3K4me2 modification at B-cell-specific EBF1 targets, which was found to be independent of transcriptional activation or repression (40). Thus, binding of EBF1 to chromatin is associated with this histone modification. A strong coincidence of H3K4 di-methylation with EBF1 occupancy can also be detected in mature B cells, although some targets bound by EBF1 specifically in mature B cells show H3K4me2 modifications already in pro-B cells (42).

Further evidence for a function of EBF1 in modulating the epigenetic landscape comes from the analysis of the *Cd79a* promoter in plasmacytoma cells expressing ectopic EBF1 (43). In pro-B, pre-B, and mature B cells, the *Cd79a* promoter is activated by the collaboration of several transcription factors including EBF1, RUNX1, E2A, and Pax-5 (43–45). In hematopoietic progenitors, plasma cells and non-B cells, the *Cd79a* promoter is methylated at CpG dinucleotides, whereas DNA methylation decreases stepwise at the onset of B-cell differentiation. EBF1 expression in plasmacytoma cells was found to induce DNA demethylation at the *Cd79a* promoter (43). Recently, Tet2 has been linked to EBF1 in a study analyzing the hypermethylation status of certain tumors, including chondrosarcomas (46). Tet2 catalyzes the conversion of 5-methylcytosine to 5-hydroxmethylcytosine and thereby initiates demethylation of DNA. Although an interaction of Tet2 and EBF1 could explain the EBF1-linked DNA demethylation, a physical or functional interaction between these proteins still needs to be determined in normal B-lineage cells.

EBF1 function has also been linked to the chromatin-remodeling complexes SWI/SNF and Mi-2/NuRD. EBF1-mediated induction of chromatin accessibility at the *Cd79a* promoter is dependent on the SWI/SNF complex, whereas the Mi-2/NuRD complex is involved in chromatin compaction and DNA hypermethylation at the *Cd79a* promoter (47, 48). However, no physical interaction between EBF1 and this complexes has been reported.

Finally, the zinc finger proteins ZNF423 and ZNF521 have been shown to interact directly with EBF1 and suppress EBF1 function (49, 50). The interaction of ZNF423 (ROAZ, Ebfaz) was mapped to a 253 amino acid region of EBF1 (aa

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240 to aa 492) including the HLH domain and was shown to inhibit EBF1-mediated transactivation in reporter assays (49). The DNA-binding motif of ZNF423 homodimers (5′-GCACCCNNGGGTGC) includes a perfect EBF1-binding site (51). Recently, aberrant ZNF423 expression has been linked to a B-cell maturation defect in B precursor acute lymphoblastic leukemia (52). The closely related ZNF521 was first identified in murine B-cell lymphoma cells and human hematopoietic progenitor cells (50, 53). Alike ZNF423, it contains 30 zinc fingers of the C_2H_2 type and negatively regulates EBF1-mediated transcription. In the hematopoietic system, ZNF521 is abundantly expressed in early progenitors, but its expression drops rapidly upon differentiation (50). Knockdown of ZNF521 in hematopoietic progenitor cells leads to enhanced B-cell differentiation with an increased number of B cells (54). A functional role for ZNF521, which includes the physical interaction with EBF1, has also been shown in adipocytes (55).

Function of EBF1 in the regulatory network of B-cell differentiation

Priming of B-cell development

Ebf1 is expressed in B-lineage cells, in adipocytes, and specific neuronal cell types (23, 26, 28, 56). EBF1 has been proposed to act as a 'pioneer' transcription factor of B-cell differentiation as it is able to induce epigenetic changes and to initiate locus activation. Increased chromatin accessibility allows for further regulation by other transcription factors and modifiers of chromatin structure. However, target site recognition by EBF1 requires a 'permissive' chromatin context. Chromatin immunoprecipitation experiments to detect binding of ectopically expressed EBF1 demonstrated that EBF1 can bind B cell-specific targets in T-lineage cells, but not in non-hematopoietic cells, such as fibroblasts (40). Moreover, genome-wide analysis of EBF1 binding in B-lineage cells and pre-adipocytic cells revealed only a small set of shared EBF1-occupied sites, indicating that only few B-cell target genes are bound in other tissues and vice versa (57, S.B. & R.G., unpublished data). A permissive hematopoietic-specific chromatin context could be established by the promiscuous transcription associated with multi-lineage priming in HSCs (58–60). The mechanism of multi-lineage priming is still obscure, but it could involve transcription factors that are expressed in HSCs (59). In addition, lymphoid-specific changes in chromatin structure may contribute to the establishment of an epigenetic landscape upon which B-lymphoid transcription factors, such as EBF1 and Pax-5, can act. Lymphoid-specific priming of chromatin and enhancers has been associated with a set of hematopoietic factors including Ikaros, Pu.1, and E2A.

Ikaros, encoded by *Ikzf1*, has multiple functions in hematopoiesis, including the regulation of self-renewal capacity of HSCs and regulation of lymphopoiesis (61, 62). This zinc finger protein can act both as an activator or a repressor of transcription by recruiting chromatin-remodeling complexes, including Mi-2/NuRD and SWI/SNF (63, 64). A knockout of *Ikzf1* leads to an arrest in LMPPs, as the cells fail to upregulate Flt3 and differentiate into CLPs (61, 65). Ikaros also contributes to the rearrangement of immunoglobulin genes by regulating the expression of *Rag* genes and mediating chromatin accessibility at the IgH and Igκ locus (66–68). Forced expression of EBF1 can partially rescue Ikaros-deficient progenitors. Although EBF1 can overcome the developmental block and promote differentiation into CD19-positive pro-B cells, it fails to restore *Rag*-dependent rearrangement of the IgH locus (66). Recently, a genome-wide binding study in pre-B cells broadened our understanding of Ikaros function in B-cell development as many of the identified target genes are associated with activation of the B-cell program. In particular, Ikaros-regulated genes are highly enriched for pre-BCR signaling, cell cycle progression, and VDJ recombination (69, 70). Interestingly, Ikaros represses the expression of PU.1 by direct binding to the *Spi1* promoter (71), and indirectly by promoting *Gfi1* expression. As Gfi1 competes with PU.1 binding at the *Spi1* promoter, Ikaros-mediated upregulation of *Gfi1* reduces PU.1 expression by interrupting an auto-regulatory loop (72).

The Ets transcription factor PU.1 is widely expressed in the hematopoietic system and performs distinct roles in the myeloid and lymphoid lineages (73). In accordance with its broad function in the hematopoiesis, PU.1-deficient mice are born alive but die within 24 h. They lack mature macrophages, neutrophils, B, and T cells, while erythrocytes and megakaryocytes are present (74). Of particular interest is the concentration-dependent function of PU.1 in the fate decision between myeloid and lymphoid lineage. High levels of PU.1 promote macrophage development, whereas low expression levels support B-cell development (75). Myeloid lineage differentiation is supported by PU.1-dependent upregulation of developmentally important cytokine receptors including *Csf1r* (76). In lymphopoiesis, a crucial step is the activation of *Il7r* by PU.1 in the CLP stage (77). In line with this finding, ectopic expression of *Il7r* in PU.1-deficient fetal liver progenitors is able to rescue early B-cell development (78).

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Interestingly, ectopic expression of EBF1 also overcomes the block of B-cell differentiation in PU.1-deficient progenitors (79). The kinetics of the EBF1-mediated rescue of differentiation is even faster than the rescue by *Il7r* expression, indicating that the developmental block in PU.1-deficient cells is only partly due to misregulation of *Il7r* and is mainly a consequence of impaired *Ebf1* expression (79). Aside from direct activation of target genes, a recent genome-wide study linked the binding of PU.1 to nucleosome remodeling and the deposition of mono-methylation at lysine 4 of histone 3 (80).

Tcf3 encodes the basic helix-loop-helix transcription factor E2A that exists in two different splice variants, termed E12 and E47. E2A-deficient mice develop normally but display increased postnatal death (81). E2A-deficient mice lack B cells due to a developmental block at the pre-pro-B-cell stage (81, 82). A more detailed analysis revealed that E2A is also needed for the maintenance of the HSC pool by restricting entry into the cell cycle (83, 84). Furthermore, E2A plays a role in lymphoid priming by supporting LMPP development from HSCs (85). This function is reflected in the reduced numbers of LMPPs in E2A-deficient mice. Strikingly, this effect seems to be dose-dependent as heterozygote animals show an intermediate reduction in LMPPs. It has been shown that E2A initiates expression of a subset of lymphoid-associated genes including *IL7r*. Interestingly, many of these E2A-dependent genes display potential binding sites for PU.1 and Ikaros in their regulatory regions, indicating a synergistic regulation (85).

The complex mechanisms of lymphoid cell priming are far from being understood and may require additional transcription factors, such as Tal1, Fli1, and Runx1 that define HSCs (60). Runx1 is of special interest as it has been linked to chromatin remodeling at the *Spi1* promoter, eventually leading to the expression of PU.1 (86). Furthermore, Runx1 was shown to recruit Tal1 and Fli1 to Runx1-binding sites (60). Miz and Myb may contribute to lymphoid priming by modulating the sensitivity to IL-7 receptor signaling (87–89), and Bcl11a appears to act prior to the expression of *Ebf1* (90). Thus, cooperation of several transcription factors and chromatin modifiers may generate conditions that are permissive for the B-lineage program and the action of the lineage-restricted transcription factors EBF1 and Pax-5.

Transcriptional regulation of *Ebf1* expression

The transition from a pluripotent lymphoid precursor to B-lineage cells is marked by the expression of *Ebf1* and *Pax-5*. In the hematopoietic system, these two transcription factors are expressed solely in the B-cell lineage and therefore, they can be considered as crucial activators of the B-cell program. *Ebf1*-deficient mice display a complete block of B lymphopoiesis at the pre-pro-B-cell stage, slightly earlier than that observed in *Pax-5*-deficient mice (91, 92). This and other observations indicate that *Ebf1* expression precedes *Pax-5* expression. Further evidence for a sequential activation came from single cell analysis of CLPs. This cell population is heterogeneous and differential expression of Ly6d distinguishes ALPs that still have B- and T-cell potential and BLPs that have lost T-cell potential (17). Moreover, fractionation of this heterogeneous population according to the expression of an *Igll1* promoter-controlled reporter gene and *Rag1* allowed the identification of three stages with different lineage potentials. Expression analysis of those sub-fractions at the single cell level indicated that many cells express *Ebf1* but lack *Pax-5* expression, underlining the sequential expression of EBF1 and Pax-5 (93).

Transcriptional activation of *Ebf1* depends on E2A encoded by *Tcf3*, which acts in concert with FoxO1 and IL-7 receptor signaling to establish expression of *Ebf1* in BLPs (94). Ectopic expression of EBF1 can, at least partially, rescue the developmental block in E2A-deficient mice (95). This observation confirms that the initiation of *Ebf1* expression is one of the major functions of E2A. Splice variant-specific deletion of E2A isoforms revealed that only E47- but not E12-deficient mice display a developmental block at the pre-pro-B-cell stage, comparable to that observed in *Ebf1*-deficient mice, suggesting that *Ebf1* is specifically activated by the E47 isoform of E2A (96).

The expression of *Ebf1* is mediated by two distinct promoters, the distal α-promoter and the stronger proximal β-promoter (97, 98). Transcription from these promoters results in the expression of two EBF1 isoforms, EBF1α and β. Due to alternative splicing, transcripts from the distal promoter lack the start codon that is used for translation of EBF1β, generating an isoform that lacks 14 N-terminal amino acids of the EBF1β isoform. However, no functional difference between the two EBF1 isoforms has yet been described. The distal α-promoter is regulated by E47 and EBF1, and it contains two consensus Ikaros-binding sites (97). Moreover, the α-promoter is regulated by IL-7 receptor signaling (98). In mice, B-cell differentiation requires IL7 receptor signaling. IL-7R-deficient mice show a developmental arrest at the pre-pro-B-cell stage and impaired *Ebf1* expression (99). Forced expression of EBF1 can partially rescue B-cell development in IL-7R-deficient mice (99). Furthermore, *Ebf1* expression can

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be restored by a constitutive active form of STAT5 that mediates a transcriptional response independent of IL-7 signaling (99). STAT5 has been shown to preferentially activate *Ebf1* from the distal promoter although no STAT5 binding can be detected at this promoter (98). Thus, IL-7 signaling may activate *Ebf1* expression indirectly by providing permissive conditions for *Ebf1* activation by other factors. Expression from the proximal β-promoter is driven by Ets1, Pax-5, PU.1, and the RUNX1/CBF-β complex (98). In the absence of Runx1, increased accumulation of the repressive histone mark H3K27me3 can be detected at the proximal *Ebf1* promoter, suggesting that Runx1 changes the epigenetic landscape at the proximal *Ebf1* β-promoter (100). Recent studies focusing on the epigenetic regulation of *Ebf1* expression identified the SWI/SNF-like BAF complex, which facilitates transcription from the proximal promoter, and MYSM1, which appears to act on both promoters (101, 102).

Once initiated, *Ebf1* expression is enforced and maintained by multiple positive regulatory feedback loops (*Fig.* 3). E2A and FoxO1 are both connected with EBF1 in a reciprocal positive feedback loop (103, 104). This regulatory unit is established by a feed-forward loop, in which E2A promotes the expression of FoxO1 (105). Moreover, both E2A and FoxO1 regulate the expression of the IL-7 receptor and therefore indirectly modulate *Ebf1* expression (85, 106). Moreover, expression of *Ebf1* is under a positive auto-regulatory feedback via the EBF1-binding site in the distal promoter. Consistent with this autoregulation, ectopic expression of EBF1 enhances to the activity of the distal but not proximal promoter (98). In *Ebf1* heterozygous mutant mice, however, *Ebf1* transcription from both promoters is reduced suggesting that the proximal promoter is indirectly regulated by EBF1, possibly via Pax-5 (98). Pax-5 binds multiple sites in the *Ebf1*β promoter, and a reciprocal positive feedback loop between EBF1 and Pax-5 is established by the EBF1-mediated activation of *Pax-5* via binding to an intragenic enhancer in the *Pax-5* locus (98, 107).

Specification of the B-cell lineage

EBF1 is a key regulator of B-cell lineage specification. Expression of EBF1 can be detected at the earliest developmental stage represented by the BLP, and it continues until the onset of plasma cell differentiation (108, 109). A conventional knockout of *Ebf1* results in a complete lack of B cells, as their development is blocked at the transition from Fr. A pre-pro-B cells to Fr. B pro-B cells (91). No rearrangement at the Igμ locus is detected *in vivo*, whereas *Ebf1*-deficient cells cultured *in vitro* show D to J_H but not V_H to DJ_H recombination (110). In heterozygous *Ebf1* knockout mice, the number of B-cell progenitors (Fr. B and C) is reduced by 50% (91). The important role of EBF1 in the specification of B-cell development was further shown by adoptive transfer experiments in which forced expression of EBF1 in wildtype bone marrow cells enriched for HSCs skewed differentiation along the B-cell pathway at the expense of other hematopoietic lineages like T cells, NK cells, and lymphoid-derived DC (111).

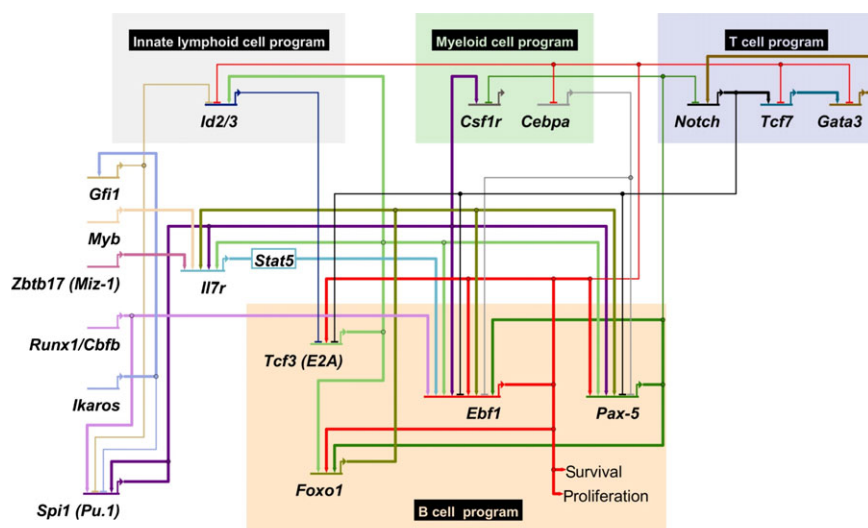

Fig. 3. Regulatory network governing early B-cell development. Key factors involved in lineage priming are depicted on the left while major transcription factors regulating B-cell specification and commitment are shown in the orange box. Genes promoting alternative lineage decisions are highlighted in gray (innate lymphoid lineage), light green (myeloid lineage), and light purple (T-cell lineage). Positive regulation is depicted with thick lines that end in an arrow, while repression is represented by thin, barred lines.

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Early biochemical studies indentified numerous key genes for B-cell differentiation, including *Cd79a*, *Cd79b*, *Cd19*, *Blk*, *Igll1*, and *Vpreb1 as* EBF1 targets (22, 112–115). Microarray analysis of EBF1-deficient CLPs validated *Pax-5* as a direct target and revealed additional EBF1 targets like *FoxO1* and *Pou2af1* (116, 117). Recent genome-wide ChIP-seq analysis in pro-B cells confirmed these target genes and extended the list to some 3000 EBF1-occupied gene loci that are enriched for genes determining B-cell identity and genes regulating the (pre-) BCR signaling cascade (40, 41). However, the regulation of EBF1 target genes requires the collaboration of EBF1 with other transcription factors. Many EBF1-regulated genes, including *Vpreb1*, *Igll1*, and *Cd79a*, also contain binding sites for E2A, and ectopic expression of EBF1 and E2A in non-B-cell lines results in a synergistic activation of the endogenous *Igll1* and *Vpreb1* surrogate light chain genes and *Igll1* reporter constructs (115, 118, 119). Moreover, a collaboration of EBF1 and E2A is underlined by the analysis of *Ebf1*$^{+/-}$*E2a*$^{+/-}$ double heterozygous mutant mice. These mice show an impaired differentiation of Fr.B pro-B cells and reduced expression of *Pax-5*, *Rag1*, *Rag2*, and *Cd79a*, whereas these phenotypes are not observed in either single-heterozygous mouse (116). Similarly, *Ebf1*$^{+/-}$*Runx1*$^{+/-}$ double heterozygous mice show more severe defects in B cell-specific gene expression and differentiation than those observed in to single-heterozygous mice (120).

Recent ChIP-seq data allowed further insight into the regulatory network. A genome-wide ChIP analysis of Ikaros in pre-B cells identified binding sites of EBF1, E2A, Pax-5, and FoxO1 in the vicinity of Ikaros-bound regions (69). In addition, PU.1 occupancy is associated with C/EBP- and AP1-binding sites in macrophages, whereas PU.1-bound regions are flanked by EBF1-, E2A-, Oct-, and NF-κB-binding sites in splenic B cells (80). Notably, a comparison of PU.1-bound regions in sequential developmental stages of B lymphopoiesis revealed a change in the composition of neighboring factor binding sites (80). In *Ebf1*-deficient pre-pro-B cells, PU.1-bound regions are primarily associated with E2A- and Runx-binding motifs, whereas PU.1 ChIP peaks are additionally flanked by EBF1- and Oct-binding sites in Rag-deficient pro-B cells. In mature splenic B cells, the association of PU.1-bound regions with Runx is reduced and an association with the NF-κB motif is gained. These observations suggest that PU.1 achieves different tasks at specific developmental stages by cooperation with different neighboring factors (80). A similar conclusion was reached by ChIP-seq analysis of E2A binding. In CLPs, a large number of E2A-occupied genes contain Ikaros- and/or PU.1-binding sites in their regulatory regions (85). Genome-wide binding assays of E2A in *Ebf1*-deficient pre-pro-B cells and *Rag1*-deficient pro-B cells revealed that E2A-bound regions are predominantly flanked by Runx-binding sites (41). In pro-B cells, E2A-bound regions were additionally flanked by EBF1- and FoxO1-binding motifs. Finally, genome-wide ChIP experiments for EBF1-bound regions in pro-B cells suggested that the EBF1-occupied regions are strongly enriched in binding sites for E2A, Pax-5, Runx, NF-κB, Stat1, Ets, and Nrf1 (40, 41). In mature B cells, many EBF1-occupied regions overlap with EBF1-bound regions in pro-B cells. However, additional EBF1-occupied sites are gained while others are lost during differentiation, resulting in a shift in the composition of neighboring binding sites between pro-B and mature B cells (42).

In addition to the integration of inputs from multiple transcription factors at *cis*-acting regions, the regulatory network underlying B lymphopoiesis also involves extensive cross-regulation of transcription factors. As outlined above, EBF1 is involved in multiple cross-regulatory feedback loops with E2A, FoxO1, and Pax-5 that help to stabilize the developmental decision of B-cell specification.

Commitment to the B-cell lineage

Besides its role in B-cell specification, EBF1 also participates in the repression of alternative cell fates, termed B-cell commitment. Early studies identified Pax-5 as a commitment factor for the B lineage because *Pax-5*$^{-/-}$ cells, which are arrested at the late pro-B-cell stage (Fr. B to Fr. C) and show rearrangement of proximal but not distal V_H segments (92, 121, 122), have acquired lineage plasticity (123, 124). Upon depletion of IL-7, *Pax-5*-deficient pro-B cells give rise *in vitro* to nearly all hematopoietic cell lineages except for B cells (123, 124). In addition, adoptive transfer of *Pax-5*$^{-/-}$ B-cell progenitors into *Rag2*$^{-/-}$ mice that lack lymphocytes allowed for a reconstitution of the T-cell compartment (125). Even mature B cells can be converted into T cells after conditional deletion of *Pax-5* via de-differentiation to uncommitted progenitors (126). Insight into the mechanism by which Pax-5 antagonizes alternative cell fates was provided by the molecular analysis of Pax-5-bound and regulated genes, which indicated that Pax-5 represses a large number of non-B cell-specific genes, including *Csf1r* and *Notch1*, which are major determinants of macrophage and T-cell development, respectively (123, 127, 128).

Several recent studies suggested an additional role for EBF1 in B-cell commitment. Analysis of the developmental

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potential of *Ebf1*-deficient lymphoid progenitors that were injected into lethally irradiated mice indicated that the *Ebf1*-deficient progenitors give rise to several lineages including myeloid, dendritic, NK, and T cells (110). Moreover, they can differentiate into T cells and myeloid cells *in vitro*, depending on the supportive conditions (110). In contrast, ectopic expression of EBF1 restricts their alternative developmental potential and promotes the generation of B cells. The lineage restriction by EBF1 is independent of Pax-5 because EBF1 expression in *Pax-5*-deficient fetal liver progenitors inhibits their myeloid and T-lineage potential *in vivo* (110). Furthermore, the myeloid differentiation capacity of *Pax-5*-deficient pro-B cells is repressed by ectopic expression of EBF1 *in vitro* (110). Likewise, enforced expression of EBF1 impedes T-cell development in *Pax-5*-deficient progenitors cultured under T-cell promoting conditions (129). Together, these results provide strong evidence for a role of EBF1 in preventing alternative lineage development in progenitors, independent of Pax-5.

Evidence for a role of EBF1 in the maintenance of B-cell identity came from experiments analyzing the developmental plasticity of *Ebf1*-deficient pro-B cells *in vivo* (130). Tamoxifen-induced deletion of floxed alleles in late pro-B cells that were adoptively transferred into alymphoid $Rag2^{-/-}Il2rg^{-/-}$ double-deficient mice allowed for the generation of CD4/CD8 double-positive T cells in the thymus and single-positive T cells in the spleen (130). Importantly, single cell analysis indicated that the peripheral T cells carried rearrangements of both the B-cell and T-cell receptor genes. In particular, the frequent detection of rearrangements using distal V_H segments confirmed that the cells were generated from Pax-5-expressing late pro-B cells because Pax-5 is required for the rearrangement of distal V_H segments. The transferred pro-B cells also carried a *Bcl2* transgene to enhance survival of cells during the process of lineage conversion. However, the limited number of B cell-specific gene rearrangements indicates that only few of the injected cells were able to convert into T cells. Aside from the conversion into T cells, *Ebf1*-deficient pro-B cells convert into innate lymphoid cells of type 2 and 3 (ILC2 and ILC3). Conversion into the myeloid lineage, however, was only rarely detected (130). Interestingly, in the bone marrow of recipient mice, a relatively large population of CD19-positive cells expressing *Pax-5* but lacking *Ebf1* expression was detected. Microarray analysis of this cell population indicated that these cells resemble lymphoid progenitors, and suggested that lineage conversion of EBF1-deleted cells occurs via de-differentiation to an intermediate progenitor-like state (130).

EBF1 antagonizes alternative cell fates by direct repression of several genes specific for alternative lineages, including *Tcf7*, *Gata3*, *Cebpa*, *Id2*, and *Id3*. *Tcf7* and *Gata3* are expressed in the earliest T-cell stages and are critical regulators of T-cell development (94). Upon induction via Notch signaling, Tcf1, encoded by *Tcf7*, promotes T-cell differentiation by the activation of T-cell fate determinants including Bcl11b and Gata3 (131). The zinc finger protein Gata3 has been proven to be essential for early T-cell development, at least in part, by regulating Notch1 expression (132, 133). Both genes, *Tcf7* and *Gata3*, are bound by EBF1, and they show increased expression upon loss of EBF1 (129, 130). EBF1 induces the repressive histone mark H3K27me3 at the *Gata3* locus (129). A direct regulation of *Gata3* by EBF1 was underlined by the expression of a synthetic zinc finger protein that blocks binding of EBF1 to a regulatory site of *Gata3*, which resulted in increased *Gata3* expression and restoration of T-lineage potential (129).

EBF1 also promotes B-cell development by repression of *Id2* and *Id3* (110, 134). ID proteins are antagonists of E proteins, including E2A, and heterodimer formation between ID and E proteins inhibits the DNA-binding ability of E proteins (135). ID2 is a major regulator of all innate lymphoid cell types (136, 137), and the lineage conversion of *Ebf1*-deficient pro-B cells into ILCs is likely accounted for by the de-repression of Id2 (130).

Finally, EBF1 also represses a key determinant of myeloid differentiation, *Cebpa*, and thereby antagonizes myeloid differentiation (110). However, CEBPα also antagonizes the expression of *Ebf1* and *Pax-5*, and ectopic expression of CEBPα in a pre-B-cell line efficiently converts the cells into a macropahage-like cell type (138). Although Ebf1 and Pax-5 both regulate B-cell lineage commitment, they appear to repress distinct genes. Notably, EBF1 mainly represses genes encoding transcription factors that determine alternative cell fates, whereas Pax-5 inhibits genes encoding receptors that promote alternative cell fates and thereby renders cells unresponsive to alternative lineage signals. By repressing distinct genes, EBF1 and Pax-5 antagonize alternative cell fates by a 'double-lock mechanism'.

Role of EBF1 in mature B cells

EBF1 is expressed not only at the onset of B-cell development but throughout B-cell differentiation until the plasma cell stage. The block in early B-cell development of *Ebf1*-deficient mice obscured an analysis of EBF1 function in mature B cells but two recent studies, using a conditional

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knockout of *Ebf1*, provided insight into the role of EBF1 in later stage B cells (42, 109). Tamoxifen-induced deletion of *Ebf1* in pre-B cells revealed an increased cell death and a defect in cell cycle progression as the mutant cells accumulate at the G1 phase (42). Notably, transformation of primary *Ebf1*-deficient pro-B cells with Abelson murine leukemia virus (A-MuLV) overcame the proliferation defect but did not rescue the survival defect. However, survival and proliferation of *Ebf1*-deficient pro-B cells is observed upon transformation by A-MuLV and ectopic expression of Myb (42).

Conditional deletion of *Ebf1* in peripheral B cells generates a strong reduction in marginal zone (MZ) B cells in the spleen, as well as B1 cells in the peritoneum (42, 109). In contrast, EBF1 seems to be dispensable for the generation and/or maintenance of follicular B (FoB) cells. However, surface expression of CD19 and CD21 on *Ebf1*-deficient follicular B cells is strongly decreased. Stimulation of *Ebf1*-deficient FoB cells showed impaired calcium mobilization and BCR signaling, including reduced phosphorylation of CD79a, CD19, and Akt (42). Moreover, BCR stimulation of *Ebf1*-deficient FoB cells results in augmented apoptosis and impaired proliferation (42, 109). Finally, *Ebf1* deletion affects germinal center (GC) B-cell development. Immunization of mice with sheep red blood cells (SRBC) initiates the differentiation of mature splenic B cells into GC B cells that perform CSR and SHM (42, 109). Although GCs are formed after immunization of mice deficient for EBF1 in mature B cells, the number of GC B cells is strongly reduced, indicating that EBF1 is required for the maintenance of GC B cells (42, 109). Thus, EBF1 plays multiple roles in the later stages of B-cell differentiation that may help to coordinate the processes of differentiation, cell proliferation, and survival.

Conclusion

Efforts aimed at understanding the regulatory circuitry that underlies B lymphopoiesis have identified several transcription factors that generate a permissive chromatin context in progenitor cells and/or determine a lineage-specific pattern of gene expression. Notably, the regulatory determinants of B lymphopoiesis are interconnected via feedback loops that stabilize lineage decisions and coordinate their action in establishing and maintaining cell type-specific patterns of gene expression. Genome-wide analysis of transcription factor occupancy has provided us an interesting insight into the combinatorial regulation of target genes. Future studies will involve single cell analysis to elucidate the heterogeneity of cell populations and the developmental capacity of individual cells. Moreover, clarification of the causal relationships of transcription factor binding, epigenetic regulation, and changes in higher order chromatin structure will be necessary to obtain a comprehensive understanding of this complex developmental process.

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