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Regulation of B cell fate commitment and immunoglobulin V_H gene rearrangements by Ikaros

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Abstract

The transcription factor Ikaros is essential for B cell development. However, its molecular functions in B cell fate specification and commitment have remained elusive. We showed that the transcription factor EBF rescued the generation of CD19⁺ pro-B cells from *Ikzf1*^{-/-} hematopoietic progenitors. Intriguingly, these pro-B cells, in spite of expressing normal amounts of EBF and Pax5, were not committed to the B cell fate. They also failed to selectively undergo V_H to DJ_H recombination at the immunoglobulin heavy-chain (*Igh*) locus. Ikaros induced V_H gene rearrangements by activating *Rag* gene expression as well as by controlling V_H gene accessibility and compaction of the *Igh* locus. Thus Ikaros is an obligate component of a network that regulates B cell fate commitment and *Igh* recombination.

Keywords

Ikaros; B cell fate commitment; *Rag* gene expression; Immunoglobulin heavy chain recombination

B lineage precursors are generated from hematopoietic stem cells (HSCs) through an ordered developmental pathway that is controlled by a hierarchical and dynamic regulatory network that includes the cytokine receptors Flt3 and interleukin 7R (IL-7R) and the transcription factors PU.1, Ikaros (A001169), Bcl11a, E2A, EBF (A000809) and Pax5 (A000403)^{1,2}. PU.1 and E2A can be regarded as competence factors for this developmental pathway as these are required for B cell development *in vivo* but their functions can be bypassed using *in vitro* culture systems and single gene molecular complementation³⁻⁶. On the other hand, EBF and Pax5 are obligate factors as their functions cannot be bypassed by simple complementation assays. These two transcription factors directly control three distinct molecular processes in early B cell development that includes activation of B lineage specific genes, repression of alternate

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lineage genes and rearrangement of the immunoglobulin heavy chain (*Igh*) locus^{1,7,8}. The molecular functions of Ikaros in the B lineage regulatory network have remained enigmatic although it has generally been assumed to function as a developmental competence factor.

Ikaros is the prototypical member of a Kruppel-like zinc finger transcription factor sub-family, which also includes Helios and Aiolos^{9,10}. The canonical Ikaros protein contains two multi-zinc finger domains: the N-terminal domain mediates sequence-specific DNA binding, while the C-terminal domain is involved in homo- or hetero-dimerization¹¹. The *Ikaros* gene (denoted *Ikzf1*) encodes multiple isoforms that are generated by alternative splicing and is widely expressed in the hematopoietic system including multipotent, self-renewing HSCs and their committed progenitors¹². Ikaros expression increases in developing lymphocytes, is maintained in granulocytes but downregulated in the macrophage and erythrocyte lineages^{12,13}. Consistent with its broad pattern of expression, Ikaros regulates various aspects of hematopoiesis. In mice, targeted disruption of Ikaros leads to a strong reduction of hematopoietic stem cell activity, a developmentally delayed and abnormal thymopoiesis as well as perturbed myelopoiesis¹⁴⁻¹⁶. The most striking phenotype associated with the loss of Ikaros is an early and complete block of B cell development^{14,15}. In the absence of Ikaros, lymphoid-primed multipotent progenitors (LMPPs) are generated but are impaired in giving rise to common lymphoid progenitors (CLPs), the latter are currently regarded as primitive B lineage progenitors^{17,18}. Due to the early nature of this block, the molecular functions of Ikaros in B lymphopoiesis remain to be delineated¹⁰. Moreover, no molecular complementation analysis using *Ikzf1*^{-/-} progenitors has been reported.

The transcription factors EBF and Pax5 play major roles in B cell fate specification and commitment by activating B lineage genes and concomitantly repressing genes of alternate lineages^{8,19,20}. EBF initiates restriction of alternate lineage options and B cell fate commitment independently of Pax5 (ref. 8). EBF induces the *Pax5* gene^{4,21}. Pax5 is required to maintain the committed state in part by functioning in a feedback loop to sustain the expression of the *Ebf1* gene^{22,23}. EBF and Pax5 appear to co-regulate (activate or repress) a number of genes and likely do so via molecular mechanisms involving both sequential and concerted action²⁴. As is the case for EBF and Pax5, Ikaros also has the dual ability to activate or repress transcription⁹. Although Ikaros family proteins have been suggested to repress surrogate light chain genes (*Igll1* and *VpreB1*) in pre-B cells²⁵, the molecular functions of Ikaros in B cell fate specification and commitment remain to be explored.

The hallmark of B cell development is the sequential DNA rearrangement of the immunoglobulin heavy and light chain loci²⁶. At the *Igh* locus, recombination is temporally ordered with rearrangement of diversity (D) and joining (J) segments preceding that of variable (V) gene segments. The latter recombination events at the *Igh* locus are specific to B lineage cells and coincide with cell fate commitment. V(D)J recombination is regulated at two major levels²⁷. One level involves the regulated expression of the recombination-activating genes 1 and 2 (*Rag1* and *Rag2*) that encode the V(D)J recombinase (A002009, A002010). The *Rag1* and *Rag2* genes are tightly linked in the genome and convergently transcribed. Distinct *cis*-acting elements and transcription factors regulate the expression of the *Rag* genes in developing B and T lymphocytes²⁸⁻³³. Ikaros proteins have been suggested to regulate *Rag* gene expression by virtue of binding sites in promoters or enhancers within the *Rag* locus. However, it remains to be determined if they do so, particularly in B lineage cells.

The second level of regulation of V(D)J rearrangement involves control of accessibility of immunoglobulin or T cell receptor gene segments to the recombinase²⁶. Both large-scale and localized molecular changes have been suggested to control the sequential and selective accessibility of different regions of the *Igh* locus to the recombinase. Accessibility and recombination of the V_H segments have been correlated with the repositioning of the *Igh* locus

from the periphery to the center of the nucleus and its compaction to generate DNA loops^{22, 34,35}. Localized events regulating accessibility include changes in chromatin structure via histone modification and the activation of germline as well as antisense transcription^{36,37}. The transcription factors STAT5 and the chromatin modifier Ezh2 regulate the accessibility of distal V_H gene segments through distinct non-redundant molecular mechanisms^{38,39}. Pax5 and more recently Yin Yang1 (YY1) have been shown to control the compaction or contraction of the *Igh* locus presumably by DNA looping^{22,40}. No role for Ikaros proteins in regulating the accessibility and recombination of the *Igh* locus has been uncovered.

Here, we showed by complementation analysis that EBF can rescue the generation of B cell precursors from *Ikzf1*^{-/-} hematopoietic progenitors thereby demonstrating that Ikaros initially functions as a B lineage competence factor. The EBF-rescued *Ikzf1*^{-/-} pro-B cells provided us with a unique tool to analyze the molecular functions of Ikaros in B cell fate specification and commitment. Our analysis indicates that Ikaros was required for B cell fate commitment as *Ikzf1*^{-/-} pro-B cells despite expressing normal amounts of EBF and Pax5 differentiated into macrophages. We also established that Ikaros plays a central role in the rearrangement of the immunoglobulin heavy chain locus at the pro-B stage by simultaneously regulating the expression of the *Rag1* and *Rag2* genes and controlling V_H gene accessibility and compaction of the *Igh* locus. These results establish that Ikaros is also an obligate regulator of B cell development. Therefore, Ikaros promotes B cell identity by repressing alternate lineage myeloid genes and by concomitantly inducing lineage-specific V_H segment recombination events at the *Igh* locus.

RESULTS

EBF rescues B cell development from *Ikzf1*^{-/-} progenitors

We initially attempted to complement *Ikzf1*^{-/-} Lin-Sca1^{hi}c-Kit^{hi} (LSK) cells with Ik-1, the largest Ikaros isoform¹¹. Under B lymphoid culture conditions, both control and Ik-1 transduced *Ikzf1*^{-/-} progenitors generated myeloid but not B lineage progeny (data not shown, see below). We next pursued functional bypass experiments to circumvent the block in B cell development caused by the loss of Ikaros. We have previously demonstrated that the transcription factor EBF is a primary B cell fate determinant⁸. It not only induces early B cell gene expression and *Igh* gene recombination but it also represses the myeloid developmental program. Wild-type and *Ikzf1*^{-/-} multipotent progenitors (LSK cells) were transduced with a control retrovirus (MIGR1) or one encoding EBF (MIG-EBF). Retroviral transduction was monitored using green fluorescent protein (GFP) whose expression was driven by an internal ribosome entry site (IRES). Following infection, the cells were plated on OP9 stromal cells with the cytokines, stem cell factor (SCF), Flt3 ligand (FL) and interleukin 7 (IL-7). After 3 days, transduced cells were sorted based on GFP expression and plated on a fresh stromal cell layer in the presence of B lymphoid cytokines (see Methods). Under these conditions, wild-type progenitors infected with control virus proliferated and differentiated after 10 days into CD19⁺ B cell precursors (Fig. 1a). As expected, expression of EBF in these cells strongly enhanced the generation of B lineage progeny⁸ (Fig. 1a).

Ikzf1^{-/-} LSK cells transduced with the control vector failed to differentiate down the B cell pathway but in contrast gave rise to Mac-1⁺ (CD11b) myeloid progeny that continued to proliferate for an extended period of time in culture (Fig. 1a). The *in vitro* expansion of Mac-1⁺ precursors caused by the loss of Ikaros is reminiscent of the phenotype observed in the bone marrow of *Ikzf1*^{-/-} mice¹⁵. Transduction of EBF in *Ikzf1*^{-/-} LSK cells resulted in the accumulation of a double negative Mac-1⁻ CD19⁻ population and blocked the generation of Mac-1⁺ myeloid progeny (Fig. 1a). Interestingly, after 2-3 weeks of culture, we reproducibly detected a small fraction of CD19⁺ B lineage progeny (Fig. 1a). Once generated, these B lineage cells were highly responsive to IL-7 and rapidly became established as the dominant cell type

in the cultures leading to the generation of homogenous cell lines. Morphological analysis by Wright staining revealed small round cells with large nuclei characteristic of lymphocyte progenitors (data not shown). Limiting dilution analysis revealed that approximately 1 in 1400 of the EBF-transduced LSK cells were able to give rise to CD19⁺ B lineage progeny (Supplementary Fig. 1 online). The generation of CD19⁺ B lineage cells from *Ikzf1*^{-/-} LSK cells was specifically dependent on EBF as retroviral transduction of E2A or Pax5 did not yield such progeny (data not shown).

The EBF-rescued *Ikzf1*^{-/-} CD19⁺ cell lines and clones isolated by limiting dilution were analyzed by flow cytometry. All clones displayed a homogeneous cell surface phenotype. In addition to CD19, they expressed CD43, CD24 (HSA), IL-7R (CD127), and c-Kit (CD117) but not Flt3 (CD135) or the myeloid markers Mac-1 and Gr-1 (Fig. 1b and not shown). We note that some clones no longer expressed GFP and retrovirally encoded EBF but nevertheless displayed an identical cell surface phenotype (Supplementary Fig. 2 online). Intriguingly Sca1, which is normally downregulated during B cell differentiation, remained highly expressed in a manner reminiscent of the phenotype of *Pax5*^{-/-} pro-B cells¹⁹ (Fig. 1b and Supplementary Fig. 2). Notably, EBF-rescued *Ikzf1*^{-/-} B lineage cells could be propagated in the presence of IL-7 and stromal cells without undergoing crisis or change of phenotype.

We next compared the gene expression profiles of the two types of EBF-rescued *Ikzf1*^{-/-} clones; type A (GFP⁺) and type B (GFP⁻) with wild-type, *Rag2*^{-/-} or *Pax5*^{-/-} pro-B cells (Fig. 1c). *Rag2*^{-/-} pro-B cells like their wild-type counterparts have undergone B cell fate specification and commitment although they retain their *Ig* loci in a germline configuration. *Pax5*^{-/-} pro-B cells are specified but not committed to the B cell fate as they mis-express alternate lineage genes. Both types of *Ikzf1*^{-/-} clones expressed the endogenous *Ebfl* and *Pax5* genes at comparable levels to wild-type cells, consistent with their CD19⁺ cell surface phenotype. Furthermore the two types of clonal derivatives expressed similar amounts of *Cd79a* (*mb-1*), *Cd79b* (*B29*), *Igll1* ($\lambda 5$) and *Vpreb1* (*VpreB*) transcripts that encode components of the pre-B cell receptor (Fig. 1c and data not shown). Finally, they also expressed the Ikaros related family member Aiolos (encoded by *Ikzf3*) that is characteristically induced in pro-B cells⁴¹. Thus, in conjunction with the cell surface phenotype analysis, these molecular data demonstrate that EBF-rescued *Ikzf1*^{-/-} CD19⁺ cells are pro-B cells. Hereafter, we refer to these cells as *Ikzf1*^{-/-} pro-B cells and focus subsequent analysis on four independent type-B clones (denoted B1 to B4) as they express wild-type amounts of endogenous EBF.

***Ikzf1*^{-/-} pro-B cells are defective in *Igh* recombination**

In spite of the similarity to wild-type cells in the expression of genes encoding the pre-B cell receptor, *Ikzf1*^{-/-} pro-B cells were strikingly defective in the expression of the recombination activating genes (*Rag1* and *Rag2*) as well as the *Dntt* gene that encodes terminal deoxyribonucleotidyltransferase (TDT) (Fig. 1c). We next analyzed the cells for D-J_H and V-DJ_H recombination events at the *Igh* locus. D-J_H recombination events could be detected in all *Ikzf1*^{-/-} clones (Fig. 1d). In contrast, no V_H to DJ_H recombination events were detected by our analysis that encompassed both proximal (V_H7183 and V_HQ52) and distal (V_HJ558) V_H genes (Fig. 1d). Cloning and sequence analysis of *Igh* regions indicated differential usage of D_H segments (Supplementary Fig.3 online). As appeared to be the case from the PCR analysis, a majority of the clones exhibited DJ_H recombination at only one allele while retaining the other in a germline configuration (Supplementary Fig.4 online). All of the DNA sequence analyses were consistent with the absence of TDT activity in the *Ikzf1*^{-/-} clones as no N-region diversity could be detected in the recombined D-J_H sequences (Supplementary Figs.3, 4). These data demonstrate that Ikaros is specifically required to promote *Rag* and *Dntt* gene expression in B lineage progenitors. In its absence, pro-B cells cannot recombine V_H gene segments within the *Igh* locus and generate pre-B cells.

***Ikzf1*^{-/-} pro-B cells retain latent myeloid potential**

We also analyzed *Ikzf1*^{-/-} pro-B cells for the expression of lineage-inappropriate genes. EBF and Pax5 repress these genes at the pro-B stage. Pax5 has been demonstrated to target the gene encoding the macrophage colony-stimulating factor receptor (*Csf1r*)^{42,43}. As shown above, both the *Ebfl* and *Pax5* genes were expressed similar to wild-type cells in *Ikzf1*^{-/-} pro-B cells (Fig. 1c). Despite normal *Ebfl* and *Pax5* expression, pro-B cells lacking Ikaros failed to properly repress the *Csf1r* gene (Fig. 1c). Interestingly, *Csf1r* expression was even higher in *Ikzf1*^{-/-} pro-B clones than in *Pax5*^{-/-} pro-B cells (3.3 fold +/- 0.5, *n* = 3). Consistent with this result, expression of CSF1R could be detected by flow cytometry on *Ikzf1*^{-/-} clones but not on *Pax5*^{-/-} cells (see below and data not shown). Mis-expression of *Csf1r* in pro-B cells in the absence of Ikaros is reminiscent of the phenotype of *Pax5*^{-/-} pro-B cells. As is the case for their *Pax5*^{-/-} counterparts⁴², *Ikzf1*^{-/-} pro-B cells mis-expressed a number of myeloid, erythroid and T lymphoid genes (Supplementary Fig. 5 online).

We next examined whether *Ikzf1*^{-/-} pro-B cells retained myeloid developmental potential. *Ikzf1*^{-/-} pro-B cells were plated onto S17 stromal cells and cultured with M-CSF (8-14 days) instead of IL-7. In parallel, wild-type, *Rag2*^{-/-} and *Pax5*^{-/-} pro-B cells were cultured under the same conditions. As previously described⁴², *Pax5*^{-/-} pro-B cells underwent a dramatic change in morphology and differentiated into macrophages that expressed Mac-1, F4/80 and high amounts of CSF1R (Fig. 2a,b). In contrast, wild-type or *Rag2*^{-/-} pro-B cells underwent considerable cell death. The residual cells maintained their B cell identity and showed no signs of myeloid differentiation based on morphology and cell surface phenotype (Fig. 2a and data not shown). Under these conditions, a substantial number of *Ikzf1*^{-/-} pro-B cells also underwent cell death but in contrast with *Rag2*^{-/-} pro-B cells, these cells like their *Pax5*^{-/-} counterparts trans-differentiated into macrophages. The differentiated cells displayed the characteristic morphology of large macrophages and expressed Mac-1, F4/80 and higher amounts of CSF1R (Fig. 2a,b). Although nearly all of the *Ikzf1*^{-/-} pro-B cell clones tested (11 out of 12) generated macrophages, the frequency of trans-differentiation of *Ikzf1*^{-/-} pro-B cells was substantially lower than that of *Pax5*^{-/-} pro-B cells (ranging between 1/60 to 1/1000, Supplementary Fig. 6 online). We note that *Ikzf1*^{-/-} pro-B cell clones displayed the ability to trans-differentiate into macrophages even after plating freshly sorted CD19⁺ cells (data not shown). Furthermore, upon exposure to M-CSF a large fraction of *Ikzf1*^{-/-} pro-B cells downregulated CD19. Thus, it appears that trans-differentiation of *Ikzf1*^{-/-} pro-B cells involves, initial loss of expression of B lineage genes followed by the acquisition of macrophage identity, the latter being an inefficient process. These results demonstrate that pro-B cells despite expressing wild-type amounts of EBF and Pax5 are not fully committed to the B cell fate in the absence of Ikaros.

Ik-1 promotes pro-B cell differentiation and commitment

To analyze the functions of Ikaros in B lineage cells we complemented *Ikzf1*^{-/-} pro-B cells with Ik-1. *Ikzf1*^{-/-} clones (type B) that did not sustain the expression of the EBF-IRES-GFP transgene were transduced with a retrovirus encoding Ik-1 (MIG-Ik-1) and monitored using GFP expression. Cells were analyzed 3 days after transduction. Ik-1 expressing cells were smaller and morphologically more similar to wild-type pro-B cells (data not shown). They maintained expression of CD43 and IL-7R but downregulated c-Kit and Sca1 in a manner that correlated with the level of Ik-1 expression (Fig. 3a). Importantly no cell surface phenotypic alterations were observed in the GFP⁻ fraction of Ik-1 cultures or in cells transduced with the control retrovirus MIGR1 (Fig. 3a). Notably, Ik-1 expression resulted in downregulation of *Csf1r* transcripts and CSF1R expression on the cell surface (Fig. 3b). Furthermore, Ik-1 inhibited the ability of *Ikzf1*^{-/-} pro-B cells to trans-differentiate into macrophages. In limiting dilution experiments, restoration of Ik-1 expression reduced the frequency of trans-differentiation to one-tenth that of cells transduced with the control vector (Supplementary Fig. 7a,b online).

Thus Ikaros appears to repress multi-lineage as well as alternate lineage hematopoietic genes during B cell differentiation and promote B cell fate commitment.

In contrast with repression of *Csf1r* transcription, Ik-1 substantially induced *Rag1* and *Rag2* gene expression (Fig. 4a). We therefore determined if Ik-1 stimulated V_H DNA rearrangements at the *Igh* locus. As shown above, *Ikzf1*^{-/-} cells transduced with the control vector harbored only DJ_H rearrangements (Fig. 4b). Expression of Ik-1 induced the rearrangement of both proximal (V_H7183 and V_HQ52) and to a lesser extent distal (V_HJ558) V_H gene segments (Fig. 4b). Thus Ikaros positively regulates V_H segment recombination at the *Igh* locus in part by controlling activation of *Rag* gene transcription in pro-B cells (see below).

Ikaros directly activates expression of the *Rag* locus

Rag gene expression is regulated during the cell cycle⁴⁴. Furthermore, we have recently shown that IL-7 signaling is a potent negative regulator of these genes in pre-B cells⁴⁵. To test if Ikaros induced *Rag* expression via modulation of the cell cycle and/or IL-7 signaling, we cultured *Ikzf1*^{-/-} clones in limiting concentrations of IL-7. Lowering the IL-7 concentration resulted in reduced cell proliferation (data not shown). Expression of *Rag1* and *Rag2* genes was assessed by QPCR. *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells were used as controls⁴⁶. As expected, *Rag* gene transcription was highly induced in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells upon attenuation of IL-7 signaling⁴⁵. In striking contrast no induction of *Rag* gene expression was observed in the *Ikzf1*^{-/-} pro-B cells (Supplementary Fig. 8 online). On the basis of these experiments, we ruled out the possibility that Ikaros regulates *Rag* gene transcription in pro-B cells by modulating the cell cycle and/or IL-7 signaling.

We next investigated whether the *Rag* locus is a direct transcriptional target of Ikaros. Several regulatory elements have been described in the *Rag* locus. In addition to the *Rag1* and *Rag2* promoters^{28,29}, there are three distinct enhancers denoted proximal enhancer (Ep), distal enhancer (Ed) and Erag³¹⁻³³ (Fig. 5a). Ed ensures lymphoid specificity while Ep and Erag are required for optimal expression of *Rag1* and *Rag2* genes specifically in developing B cells. Each of these elements contains several potential Ikaros binding sites. To determine whether Ikaros binds to these regulatory regions *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays using *Rag2*^{-/-} pro-B cells. We note that a portion of the *Rag2* coding exon is deleted in these cells⁴⁷ and they express wild-type amounts of *Rag1* transcripts. The pericentromeric γ -satellite repeat sequences (γ -sat.) were used as a *bona fide* Ikaros target in these ChIP assays⁴⁸ while chromatin from *Ikzf1*^{-/-} mutant pro-B cells served as a negative control. Ikaros binding was observed at multiple regulatory elements of the *Rag* locus with the notable exception of the *Rag2* promoter (Fig. 5b). Electrophoretic mobility-shift assays (EMSA) demonstrated the presence of Ikaros binding sites in the different regulatory elements analyzed by ChIP (Fig. 5c and Supplementary Fig. 9 online). To further analyze the molecular consequences of Ikaros binding to the *Rag* locus, we assessed the histone modification status of these regulatory regions in pro-B cells lacking or expressing Ikaros. The degree of histone-H3 acetylation was analyzed by ChIP assays in *Ikzf1*^{-/-} pro-B cells and compared with that observed in *Rag2*^{-/-} pro-B cells. Strikingly none of the *Rag* locus regulatory domains displayed this active chromatin modification in the absence of Ikaros (Fig. 5d). Thus Ikaros directly targets the *Rag* locus and appears to induce histone acetylation while activating *Rag* gene transcription.

To reinforce the above conclusion, we tested if increased expression of Ikaros leads to upregulation of *Rag1* and *Rag2* expression in pro-B cells. *Pax5*^{-/-} pro-B cells were transduced with MIG-Ik-1 retrovirus or the control vector. Transduced cells were sorted based upon GFP expression and Q-PCR was used to analyze *Rag* gene transcripts. Increased amounts of Ik-1 augmented *Rag1* (2.9 fold) and *Rag2* (5.6 fold) gene expression (Supplementary Fig. 10 online). Notably, the Ik-7 isoform that cannot bind DNA failed to upregulate *Rag* gene

expression. Thus Ikaros regulates *Rag* gene expression in a concentration dependent manner in pro-B cells.

Ikaros controls accessibility and compaction of *Igh* locus

To determine if the molecular defect in V_H gene recombination in *Ikzf1*^{-/-} pro-B cells (Fig. 1d, 4b) was simply due to a failure to properly activate the *Rag* locus, we restored *Rag* gene expression in these cells. For these experiments we made use of retroviruses expressing RAG1 (RAG1-IRES-Dsred, denoted MIRR-Rag1) and RAG2 (RAG2-IRES-GFP, denoted MIGR-Rag2). Dsred, GFP doublepositive cells were FACS sorted after stepwise infection with the two retroviral vectors, expanded in culture in the presence of IL-7 and analyzed for DNA rearrangements at the *Igh* locus. As previously shown, cells transduced with control vectors did not recombine either proximal or distal V_H segments. Expression of *Rag1* along with *Rag2* modestly stimulated V_H to DJ_H rearrangements involving the proximal (V_H7183 and V_HQ52) V_H segments (Fig. 6a, 4b). These data suggest that in the absence of Ikaros, RAG proteins cannot efficiently access the V_H domain of the *Igh* locus. To further explore this possibility, we examined germline transcription across the *Igh* locus as an indicator of chromatin accessibility. Germline transcripts that initiate within the heavy-chain intronic enhancer, from the DQ52 segment promoter or from the proximal V_H7183 , V_HQ52 promoters were unaffected by the loss of Ikaros (Fig. 6b). In contrast, germline transcripts from the distal V_HJ558 gene segment promoters were substantially reduced in the *Ikzf1*^{-/-} cells. Thus, Ikaros appears to promote the accessibility of V_H gene segments to recombination, particularly that of the distal V_HJ558 gene segments (see below).

Repositioning away from the nuclear lamina and large-scale compaction are two nuclear events that have been proposed to regulate the ability of the distal V_H genes to undergo recombination^{22,34,35}. Three-dimensional fluorescence *in situ* hybridization (3D Immunofluorescence) was used to analyze the nuclear disposition and compaction of *Igh* alleles in *Ikzf1*^{-/-} pro-B cells. *Rag2*^{-/-} pro-B and EL-4 T cells were used as controls. Unlike in EL-4 T cells, the *Igh* alleles in *Ikzf1*^{-/-} pro-B cells were positioned away from the nuclear lamina similar to their disposition in *Rag2*^{-/-} pro-B cells (Fig. 7a and Supplementary Fig. 11 online). We next analyzed compaction of the *Igh* locus by measuring the distances between distal V_HJ558 genes and the constant C_H regions on the same allele. As previously described³⁹, *Igh* alleles in EL-4 nuclei demonstrated a wide range of intralocus distances while *Rag2*^{-/-} pro-B cells displayed a more constrained distribution (Fig. 7a,b). The profile of *Igh* alleles in *Ikzf1*^{-/-} pro-B cells resembled that of EL-4 T cells and not of *Rag2*^{-/-} pro-B cells (Fig. 7a,b). The intralocus distance distributions of *Ikzf1*^{-/-} and EL-4 cells were not significantly different from each other ($P > 0.03$) while both were statistically different from that of *Rag2*^{-/-} cells ($P < 0.0001$ and $P = 0.0003$, respectively). Thus Ikaros is not required for repositioning of *Igh* alleles away from the nuclear lamina. Instead, it seems to regulate spatial constraints on the *Igh* locus that are manifested in its apparent compaction. Collectively, these results reveal Ikaros to be a regulator of both accessibility and compaction of the *Igh* locus.

DISCUSSION

Recently we have proposed that the transcription factor EBF functions as the primary determinant of the B cell fate^{1,49}. EBF is able to rescue B cell development *in vitro* from PU.1-, EBF- or E2A-deficient progenitors^{4,5,8}. Herein, we demonstrate that EBF is also able to restore the B cell developmental potential of *Ikzf1*^{-/-} progenitors. However, the generation of CD19⁺ cells was significantly delayed compared to the aforementioned experiments. Limiting dilution experiments demonstrate that the delayed kinetics reflect a low frequency of *Ikzf1*^{-/-} LSK progenitors that can be rescued with EBF. We suggest that this is due to the fact that a large proportion of the LSK compartment in absence of Ikaros is poised for myeloid

differentiation¹⁸. Consistent with this view, *Ikzf1*^{-/-} LSK progenitors undergo rapid myeloid differentiation even when cultured in B lymphoid inducing conditions. Moreover the *Ikzf1*^{-/-} LSK cellular compartment is characterized by upregulation of myeloid cell fate determinants and a failure to induce expression of early B cell genes¹⁸ (C. Spooner and H.S., unpublished data). EBF's ability to rescue *Ikzf1*^{-/-} cells is reminiscent of its ability to redirect at low frequency myeloid progenitors toward the B lymphoid lineage⁸. When ectopically expressed in myeloid progenitors EBF not only induces the B cell program but also represses myeloid gene expression. It is notable that expression of EBF in *Ikzf1*^{-/-} LSK cells promotes the proliferation of a progenitor population that is Mac-1⁻ CD19⁻. These results are consistent with the ability of EBF to antagonize myeloid determinants such as PU.1 and C/EBPα⁸.

It has been proposed that Ikaros is essential for activating the *Flt3* and *Il7r* genes that encode two key cytokine receptors for lymphoid differentiation^{1,16,50}. The block to B cell development in *Ikzf1*^{-/-} mice has been suggested to be due to deficiencies in these two signaling pathways. We tested this possibility by restoring expression of Flt3 or IL-7R in *Ikzf1*^{-/-} LSK cells (data not shown). We were unable to functionally bypass the requirement for Ikaros in B cell development with either of these cytokine receptors. Although it remains possible, that co-expression of Flt3 and IL-7R may rescue B cell development from *Ikzf1*^{-/-} LSK cells, our results are consistent with the demonstration that lymphoid-primed multipotent progenitors can be generated in absence of Ikaros¹⁸. Our analysis of *Ikzf1*^{-/-} pro-B cells rescued with EBF indicates that Ikaros is not strictly necessary for the expression or functioning of IL-7R. Collectively, these results weaken the argument that Ikaros promotes B cell fate specification by controlling the expression of Flt3 and/or IL-7R. The successful rescue of *Ikzf1*^{-/-} progenitors by EBF suggests instead that Ikaros is required for the developmental induction of the *Ebf1* gene. In the future, it will be important to determine if the *Ebf1* gene is an Ikaros target as is the case for the *Rag* genes (see below).

One of the primary functions of Ikaros in LMPPs may be to antagonize the myeloid program. Intriguingly, rescued *Ikzf1*^{-/-} pro-B cells despite the normal expression of EBF and Pax5, are unable to properly repress alternate lineage genes including *Csf1r*. Thus we propose that Ikaros functions to attenuate the *Csf1r* gene in LMPPs but also to maintain its repression in pro-B cells. *Ikzf1*^{-/-} pro-B cells are not committed to the B lineage as they are able to differentiate into macrophages upon culturing in M-CSF. This result predicts that conditional deletion of *Ikzf1*, the gene encoding Ikaros, at the pro-B cell stage should also result in loss of B cell commitment. We propose that Ikaros ensures B cell fate commitment by maintaining the repressed state of alternate lineage genes. Thus, B cell fate commitment is not simply a consequence of the action of a single transcription factor⁷ but instead dependent on an elaborate network of regulatory molecules that include EBF, Pax5 and now Ikaros⁸. These factors are likely to function in both a sequential and concerted manner during B cell development from LMPPs to restrict alternate cell fate options ultimately leading to commitment.

How might Ikaros function to repress myeloid genes? Several molecular mechanisms of repression have been described in the case of Ikaros. One is linked to its ability to associate with histone deacetylase (HDAC)-containing complexes NURD or Sin3 (ref. 9). In favor of this hypothesis, we note that the *Csf1r* locus exhibits a high degree of histone H3 acetylation in *Ikzf1*^{-/-} pro-B cells unlike its status in Ikaros expressing *Rag2*^{-/-} pro-B cells (data not shown). Another possible mechanism involves a competition on overlapping binding sites as described with EBF in the *Igll1* (γ15) locus or with Ets1 in the *Dntt* (TdT) locus^{25,51}. A third mechanism involves the proposed ability of Ikaros to associate genes with pericentromeric heterochromatin^{48,52}. Our results demonstrate similarities as well as differences between *Ikzf1*^{-/-} and *Pax5*^{-/-} pro-B cells. Both express c-Kit and Sca1 and numerous alternate lineage genes^{19,42}. Specifically, they fail to repress the *Csf1r* gene and are not committed to the B

lineage and can differentiate into macrophages⁴². Notably, as recently described for EBF⁸, raising the concentration of Ikaros in *Pax5*^{-/-} pro-B cells also leads to the repression of the *Csf1r* locus and restrains the ability to these cells to differentiate into macrophages (data not shown). Although EBF or Ikaros can bypass the requirement for Pax5 in repression of the *Csf1r* gene in *Pax5*^{-/-} pro-B cells, we suggest that in wild-type pro-B cells such repression is mediated by concerted action of the three transcription factors. Interestingly, *Ikzf1*^{-/-} pro-B cells, in contrast to their *Pax5*^{-/-} counterparts, failed to undergo T cell differentiation *in vitro* or *in vivo* (data not shown). Exposure of *Ikzf1*^{-/-} pro-B cells to the Notch-ligand Delta 1 induced expression of the Notch target genes *Hes1* and *Dtx1* and resulted in rapid apoptosis (data not shown). Thus *Ikzf1*^{-/-} pro-B cells can differentiate into macrophages but not T lineage cells. We demonstrate that Ikaros specifically controls the activation of the *Rag* locus and subsequently the transition between the pro- and pre-B cell stages that requires the rearrangement of the *Igh* locus. These data are consistent with the analysis of a mouse strain expressing a hypomorphic allele of Ikaros (*Ikzf1*^L) generated by insertion of *lacZ*⁵³. These mice accumulate B220⁺CD43⁺IgM⁻ cells, which comprise the pro-B and early pre-B compartment. The authors noted decreased expression of the *Rag1*, *Rag2* and *Dntt* genes in B220⁺IgM⁻ cells but it was unclear if this was due to a defect in pro-B cells. Our results suggest that a majority of the *Ikzf1*^{L/L} B220⁺CD43⁺ cells are pro-B cells that have failed to recombine the *Igh* locus.

We show that Ikaros binds *in vitro* and *in vivo* to multiple sites within the *Rag* locus and regulates histone acetylation as well as transcription. As reported for Foxp1⁵⁴, Ikaros targets the most 5' distal enhancer designated Erag. Consistent with our findings, B lineage progenitors in Erag deficient mice are specifically impaired in V_H-to-DJ_H rearrangements³². We note that loss of Ikaros leads to a more profound block in the expression of the *Rag* genes than deletion of Erag. This result is consistent with our data indicating that Ikaros binds other regulatory elements in the *Rag* locus such as the proximal and distal enhancers as well as the *Rag1* promoter. How are *Ikzf1*^{-/-} progenitors able to undergo D_H-to-J_H recombination? We suggest that a low level of *Rag* gene expression in lymphoid-primed multipotential progenitors that is independent of Ikaros may be sufficient to promote monoallelic D_H-to-J_H recombination. It is important to note that the *Rag* genes represent a rare example of targets that are activated by Ikaros. Previously Ikaros has been shown to bind and activate the *Cd8a* gene in T lineage cells⁵⁵. As is the case for the *Cd8a* locus, the molecular mechanism by which Ikaros activates transcription of the *Rag* locus remains to be determined. Ikaros regulates histone acetylation at the *Rag* locus and may do so by recruiting chromatin-modifying complexes⁹. Our results indicate that Ikaros also positively regulates the expression of the *Dntt* gene in pro-B cells that is required for addition of non-templated nucleotides at coding joints within the assembled Ig segments. The *Dntt* gene promoter contains Ikaros binding sites that have been implicated in repression of the *Dntt* gene during differentiation of CD4⁺CD8⁺ thymocytes⁵¹. It is possible that Ikaros is able to activate the *Dntt* gene through these sites in the promoter at an earlier stage in T and B cell development. Finally, we note that Ikaros is not required for the expression of the *Rag* genes in T lineage cells. This dispensability could be due to replacement of Ikaros function by alternate members of the family or usage of other *cis*-regulatory elements in the *Rag* locus during T cell development.

Restoration of *Rag* gene expression in *Ikzf1*^{-/-} pro-B cells modestly induces recombination of proximal V_H gene segments. In contrast expression of Ik-1 in these cells promotes more efficient proximal V_H gene recombination as well as limited recombination of distal V_H genes. Although, it is possible that these differences in the efficiency and recombination bias of V_H gene segments is due to differing concentrations of Rag protein expression, it is unlikely given that reduced Rag expression in *Foxp1* or Erag mutant B lineage cells does not result in preferential usage of proximal V_H genes. We suggest that the aforementioned differences are due to the fact that Ikaros functions in a dual capacity. It not only activates *Rag* gene expression

but also promotes V_H genes accessibility. Consistent with the latter possibility, germline transcription of the distal V_H gene segments is compromised in *Ikzf1*^{-/-} pro-B cells. Ikaros is unlikely to be regulating germline transcription of the distal V_H segments by modulating IL-7 signaling and Stat5 activity as the *Ikzf1*^{-/-} pro-B cells proliferate normally in response to IL-7³⁹. Although putative Ikaros binding sites have been described in the V regions of the *Igh* locus⁵⁶ it remains to be determined if Ikaros regulates transcription and recombination accessibility via binding to these sites. At the level of nuclear compartmentalization, Ikaros is not required for repositioning of the *Igh* locus away from nuclear lamina. In contrast, Ikaros controls compaction of the *Igh* locus that has been proposed to promote the rearrangement of the distal V_H genes. Compaction of *Igh* locus in *Ik*^{-/-} pro-B cells is similar to that observed in *Pax5*^{-/-} or *Yy1*^{-/-} pro-B cells. However, since *Ikzf1*^{-/-} pro-B cells express wild-type amounts of *Pax5* and *Yy1* transcripts, the reduced *Igh* locus compaction phenotype in these cells cannot be attributed to impaired expression of *Pax5* or *Yy1* (data not shown). Furthermore, the defect in germline transcription of distal V_H genes observed in *Ikzf1*^{-/-} pro-B cells argues for an independent mechanism. Notably, in contrast with our findings that demonstrate Ikaros as a positive regulator of *Igh* recombination, Ikaros appears to promote the association of the *Igh* locus with pericentromeric heterochromatin during allelic exclusion^{57,58}. The molecular mechanisms underlying these distinct Ikaros functions during B cell differentiation remain to be explored.

Collectively, our results reveal key, non-redundant molecular functions of Ikaros during B cell development. Based on the findings that, Ikaros function in multipotent progenitors can be partially by-passed by the expression of EBF, we propose that Ikaros acts in LMPPs as a B lineage competence factor both by restraining the myeloid developmental program and by enabling the expression of EBF that is a primary B cell fate determinant. Moreover, we show that Ikaros is an obligate regulator of the early molecular program that is operative in pro-B cells. In this context Ikaros functions in a non-redundant manner to establish B cell identity by repressing alternate cell fate options. Our results suggest that commitment to the B lineage requires the sequential and concerted actions of Ikaros, EBF and Pax5. Finally, Ikaros is essential for positively regulating V_H to D_{J_H} rearrangement at the *Igh* locus that is the defining hallmark of B cell fate commitment. Importantly Ikaros regulates this DNA recombination step by activating the expression of the *Rag* genes and by controlling the accessibility as well as compaction of the *Igh* locus.

METHODS

Retroviral vectors

The murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-GFP, MIGR1) retroviral vector has been described previously³. The EBF, *Ik-1* and *Ik-7* vectors are derivatives of MIGR1 (refs. 4,59). *Rag1* and *Rag2* coding sequences were amplified by PCR from pEBB-*Rag1* and pEBB-*Rag2* plasmids (gifts from D.G. Schatz, Yale University, New Haven, CT) and inserted into *XhoI* and *HpaI* cloning sites of MIR1 (MSCV-IRES-Dsred) and MIGR1 respectively. Retroviruses were produced by transient transfection in Plat-E packaging cell line as described previously⁴.

Isolation, retroviral transduction and differentiation of *Ikzf1*^{-/-} hematopoietic progenitors

Ikzf1^{-/-} mice as well as wild-type control littermates were bred and maintained in specific pathogen-free conditions with oral antibiotics at the Animal Facilities of the University of Chicago. Mice used for this study were between 3 and 6 weeks old. Mouse handling involved protocols that were approved by the Institutional Animal Care and Use Committee. Hematopoietic progenitors were enriched from bone marrow cell suspensions by magnetic depletion (Miltenyi Biotec) using lineage-specific antibodies. The LSK population was

isolated, by cell sorting, based on the expression of c-Kit and Sca1. LSK cells were suspended in retroviral supernatants with 12.5 mg/ml polybrene and centrifuged 1,400 g, at 30 °C, for 2 h. The cells were then washed and plated on OP9 stromal cells in OptiMem medium (Invitrogen) supplemented with 5% fetal calf serum (FCS) in presence of SCF (10 ng/ml), FL (10 ng/ml), and IL-7 (5 ng/ml). After 72 h, transduced cells were sorted based on GFP expression and re-plated under the same culture conditions. Following sequential withdrawal of FL and SCF upon two-day intervals, cultures were maintained in IL-7. Medium was changed twice a week and cells were re-plated on fresh OP9 stroma once a week. Cellular differentiation was monitored on a weekly basis by flow cytometry.

Propagation and retroviral transduction of pro-B cells

Wild-type or *Rag2*^{-/-} pro-B cells were propagated on OP9 stromal cells in OptiMem medium supplemented with 5% FCS and IL-7 (5 ng/ml). *Pax5*^{-/-} pro-B cells (a gift from M. Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) were cultured according to a previously described protocol⁴². Cell lines were transduced with retroviral constructs as described above for primary cells. Transduced cells were sorted based on GFP expression and propagated in culture.

Assaying myeloid potential of pro-B cells

Wild-type or mutant (*Ikzf1*^{-/-}, *Rag2*^{-/-} or *Pax5*^{-/-}) pro-B cells were transferred onto S17 stromal cells and initially cultured in α -MEM medium supplemented with 10% FCS and IL-7 (5 ng/ml). After two days, the cells were switched to myeloid culture conditions by replacing the above medium with α -MEM supplemented with 10% FCS and M-CSF (20 ng/ml).

Antibodies and Flow cytometry

Cell suspensions were stained using standard procedures. Cells were analyzed on a FACSCalibur using CellQuest or FlowJo software (Becton Dickinson). Cells were sorted at the Flow cytometry facility of the University of Chicago using MoFlo-HTS (DakoCytomation) or FACS Aria (Becton Dickinson) cell sorters. The antibodies used in this study are listed in Supplementary Table 1 online.

Gene expression analysis

RNA was isolated using TRIzol LS reagent (Invitrogen) and reverse-transcribed with random hexamers and the SuperScriptTM II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For semi-quantitative analysis the reverse-transcription reaction products were serially diluted and specific transcripts were amplified by PCR. RT-PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For quantitative analysis, real-time RT-PCR was performed using Brilliant SYBR Green and the Mx4000 system (Stratagene). Each RNA sample was normalized based on *Hprt1* expression. Sequences of primers used for semi-quantitative and quantitative-PCR are provided in Supplementary Tables 2 and 3 online, respectively.

PCR analysis of Igh rearrangements

DNA recombination at the *Igh* locus was assessed as described previously³⁹. Serial dilutions of genomic DNA were analyzed by PCR with primers specific for V_HJ558, V_H7183 or D_H segments and a primer 3' of JH3 (Supplementary Table 4 online). PCR products were separated by electrophoresis on a 1% agarose gel and analyzed by Southern blotting with a DHFL16-JH4 probe.

Chromatin Immunoprecipitation assays (ChIP)

Chromatin crosslinking and Immunoprecipitation was performed as described previously³⁹. The following antibodies were used; anti-acetylated Histone H3 (06-599; Upstate Biotechnology), anti-Ikaros-Ct (rabbit antiserum generated against a carboxy terminal fragment of Ikaros⁶⁰) and control IgG (sc-2027; Santa Cruz). Immunoprecipitated DNA sequences were analyzed by quantitative real-time PCR as described above. Primers used in ChIP assays are listed in Supplementary Table 5 online.

Electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were prepared from 293T cells transfected with the Ik-1 constructs cloned into the pcDNA3 expression vector as previously described⁴⁸. Negative controls included nuclear extracts from cells transfected with vector alone or the Ikaros-159A mutant construct⁴⁸. Ikaros expression was verified by Western blotting (data not shown). DNA binding probes were generated by annealing synthetic double-stranded oligonucleotides and end-labeling using polynucleotide kinase and ³²P-labeled γ -ATP. Sequences of the probes are indicated in Supplementary Table 6 online. Ik-bs4 probe contains a high affinity consensus Ikaros binding site^{11,48}. The anti-Ikaros-Ct serum described above for ChIP assays was used for supershifting Ikaros protein-DNA complexes. Pre-immune serum was used as negative control.

Three-dimensional fluorescence *in situ* hybridization (3D Immuno-FISH)

3D Immuno-FISH experiments were performed as previously described³⁹. DNA probes were made by nick translation of bacterial artificial chromosome clones corresponding to the V_HJ558 and heavy-chain constant (C_H) region of the *Igh* locus. Specific DNA sequences were detected with avidin-fluorescein isothiocyanate or mouse anti-digoxin-indocarbocyanine (Jackson ImmunoLabs). The nuclear lamina was demarcated by anti-Lamin B1 staining. Image analysis was performed using ImageJ software. At least 100 signal pairs for each cell type were assigned scores for analysis. The unpaired *t*-test was used for the statistical analysis of the intralocus distances.

Accession numbers

Ikaros; <http://www.signaling-gateway.org/molecule/query?afcsid=A001169> EBF;
<http://www.signaling-gateway.org/molecule/query?afcsid=A000809> Pax5;
<http://www.signaling-gateway.org/molecule/query?afcsid=A000403> Rag1;
<http://www.signaling-gateway.org/molecule/query?afcsid=A002009> Rag2;
<http://www.signaling-gateway.org/molecule/query?afcsid=A002010>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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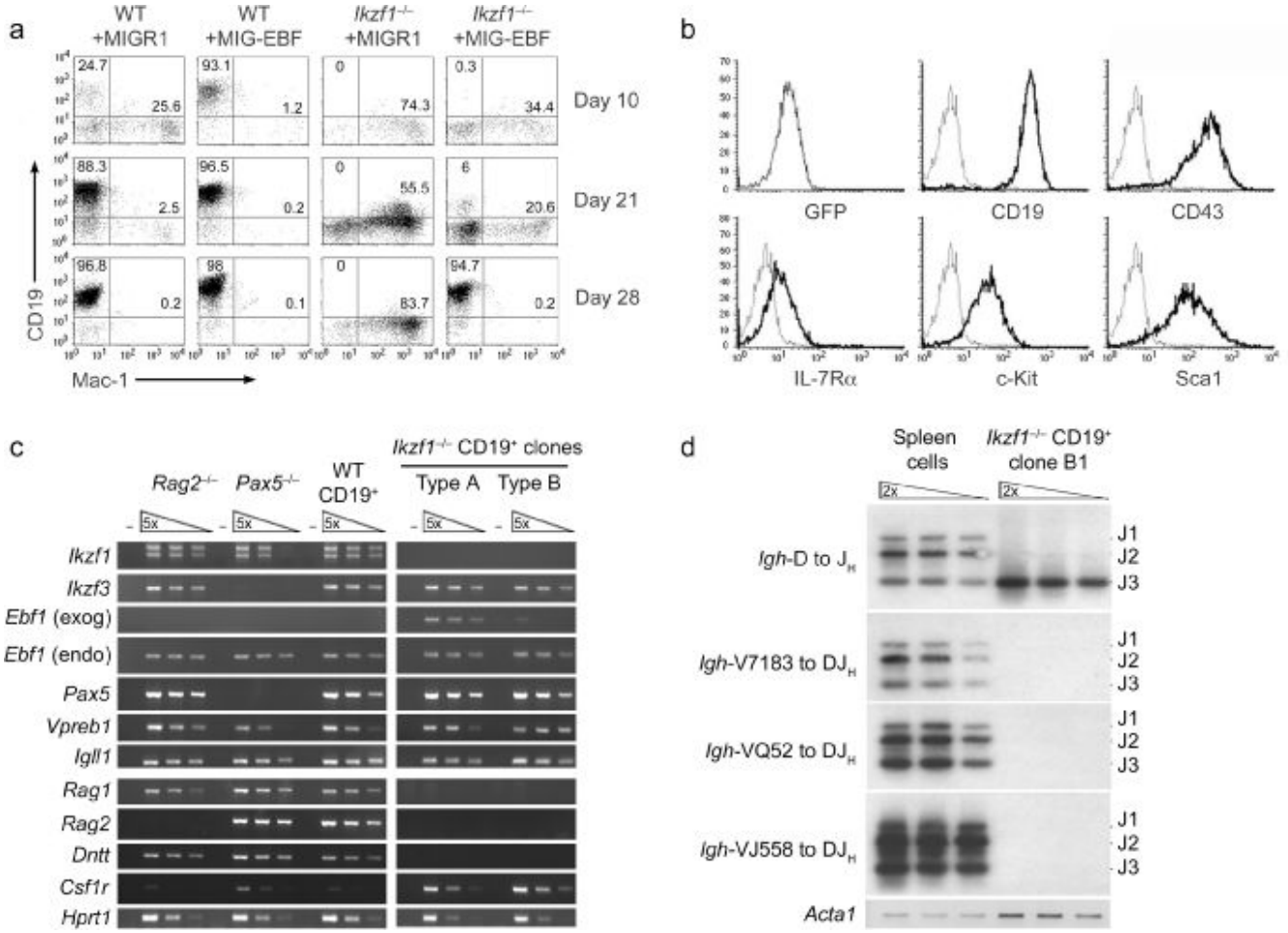


Figure 1. EBF expression restores B-cell development from *Ikzf1*^{-/-} hematopoietic progenitors
(a) Kinetic analysis of B cell development induced by the expression of EBF in *Ikzf1*^{-/-} LSK progenitors. Wild-type or *Ikzf1*^{-/-} LSK progenitors were transduced with MIGR1 or MIG-EBF viruses and cultured on OP9 stromal cells under B lymphoid conditions. Cultures were harvested at day 10, 21 and 28 and analyzed for generation of B lymphoid (CD19⁺) or myeloid (Mac-1⁺) precursors. **(b)** Flow cytometry of an *Ikzf1*^{-/-} CD19⁺ clonal cell line rescued with EBF. **(c)** Gene expression analysis of *Ikzf1*^{-/-} CD19⁺ clones. Type A clones express both exogenous (exog) and endogenous (endo) *Ebf1* genes whereas type B clones only express the latter. Serial dilutions of cDNA were analyzed by RT-PCR after normalization to *Hprt1*. cDNAs from wild-type CD19⁺ cells generated *in vitro* (denoted WT CD19⁺) as well as *Rag2*^{-/-} and *Pax5*^{-/-} pro-B cell lines were used as controls. **(d)** PCR analysis of *Igh* rearrangements. Genomic DNA from *Ikzf1*^{-/-} CD19⁺ clone B1 was analyzed using primers that detect D_H to J_H or V_H7183-, V_HQ52- and V_HJ558-to-DJ_H rearrangements. PCR products were detected by Southern blotting. Genomic DNA from spleen cells was used as a control. DNA samples were normalized with *Acta1* (encoding α -actin). All data are representative of at least two independent experiments.

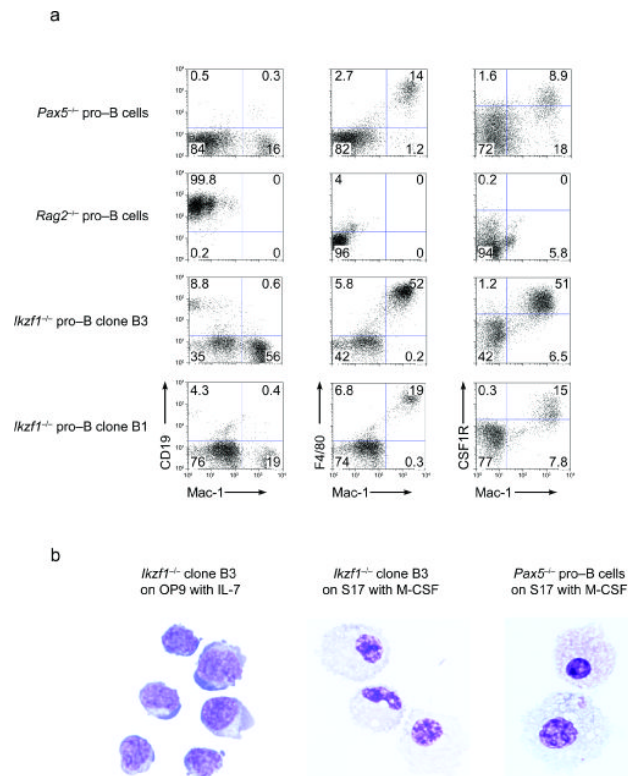


Figure 2. *Ikzf1*^{-/-} pro-B cells can trans-differentiate into macrophages

Pax5^{-/-}, *Rag2*^{-/-} and *Ikzf1*^{-/-} pro-B cells were plated on S17 stromal cells under B lymphoid conditions in the presence of IL-7 for 2 days and then switched to myeloid culture conditions in the presence of M-CSF. **(a)** Flow cytometry of switched cultures (day 12) examining expression of CD19, Mac-1, F4/80 and CSF1R. **(b)** Cellular morphology of Mac-1⁺ cells generated under the switched culture conditions. *Pax5*^{-/-} or *Ikzf1*^{-/-} cells were sorted on the basis of Mac-1 expression and examined by Wright staining. *Ikzf1*^{-/-} pro-B cells cultured on OP9 in the presence of IL-7 were used to compare morphology. All data are representative of three independent experiments.

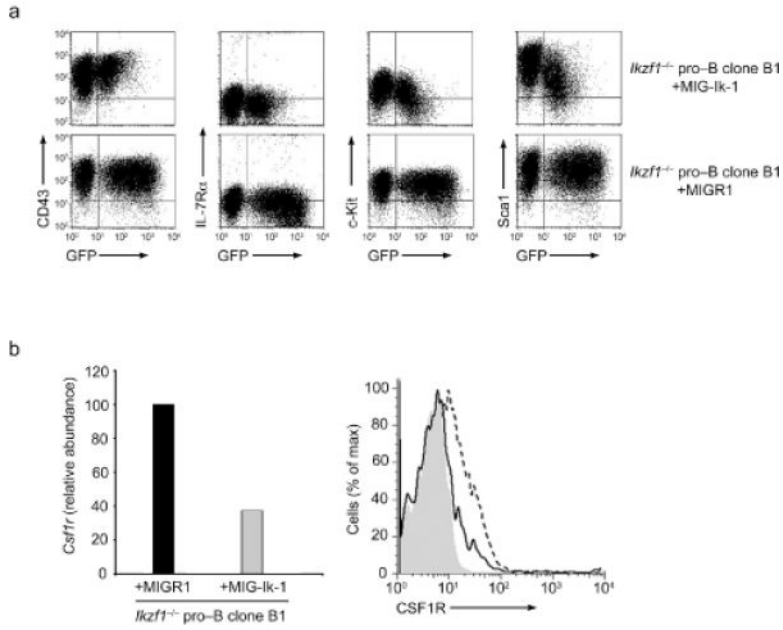


Figure 3. Restoration of Ik-1 expression in *Ikzf1*^{-/-} pro-B cells promotes B-lineage commitment
Ikzf1^{-/-} CD19⁺ clones were transduced with a control vector (MIGR1) or a derivative encoding Ik-1 (MIG-Ik-1) and analyzed 3 days after transduction. **(a)** Flow cytometry examining expression of indicated cell surface markers in Ik-1 transduced (top) and control (bottom) cells. Transduced cells are distinguished on the basis of GFP expression. **(b)** Left, relative expression of *Csf1r* transcripts in *Ikzf1*^{-/-} CD19⁺ cells transduced with MIG-IK-1 or MIGR1 (set to 100). Right, flow cytometry of CSF1R protein on the surface of *Ikzf1*^{-/-} CD19⁺ cells transduced with MIGR1 (dashed line) or MIG-Ik-1 (solid line). Isotype control is shown in grey. Analyses are gated on GFP^{hi} cells. All data are representative of three independent experiments.

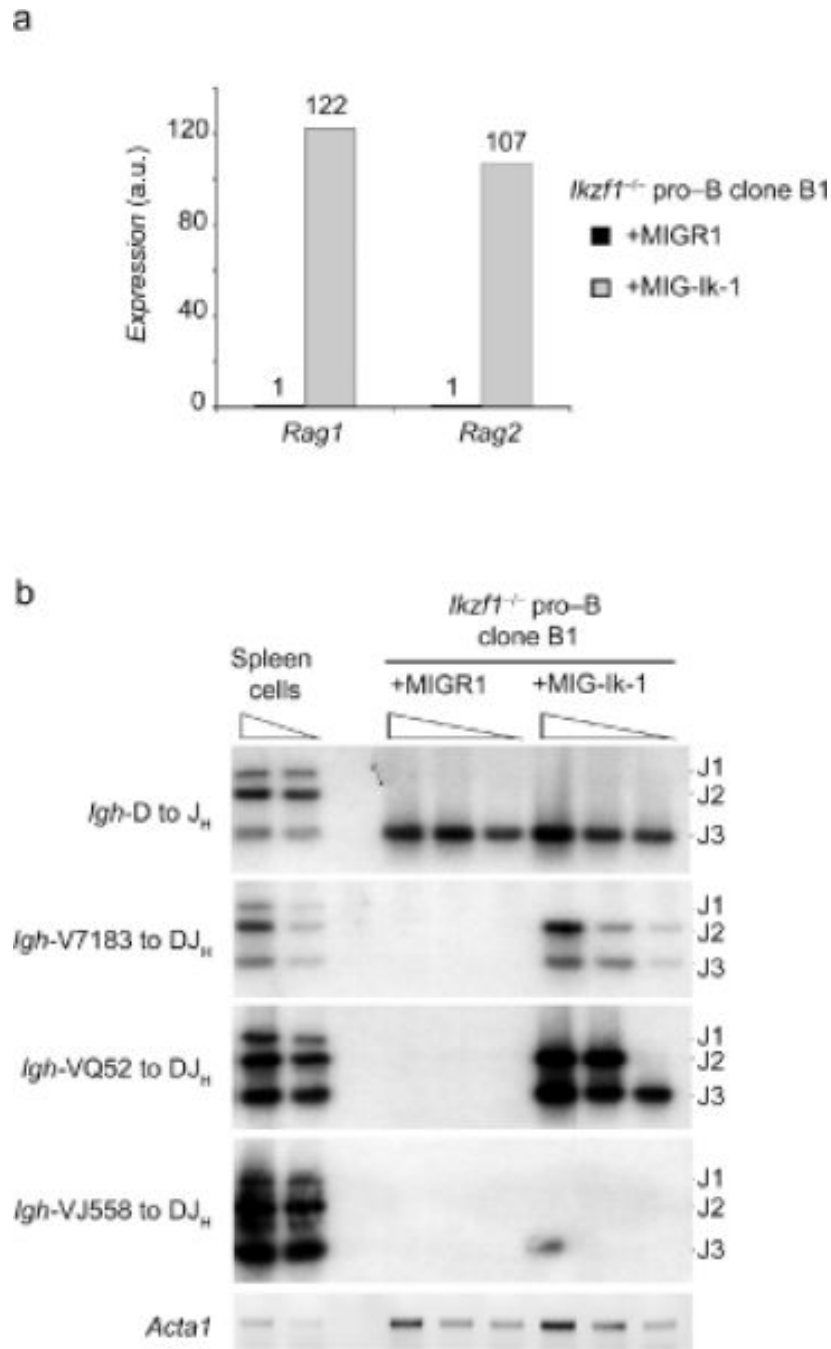


Figure 4. Restoration of Ik-1 expression in *Ikzf1*^{-/-} pro-B cells induces *Rag* gene expression and V_H to DJ_H rearrangements
(a) Q-PCR analysis of *Rag1* and *Rag2* mRNA expression in *Ikzf1*^{-/-} cells transduced with MIGR1 or MIG-Ik-1 vectors. *Hprt1* transcripts were used for normalization. The relative expression of each gene is expressed as a fold difference in relation to that detected in MIGR1 transduced cells (set to 1). **(b)** PCR analysis of *Igh* rearrangements. Genomic DNA from *Ikzf1*^{-/-} pro-B cells transduced with MIGR1 or MIG-Ik-1 was analyzed as described in Fig. 1d. Genomic DNA from the mouse spleen was used as a control. All data are representative of three independent experiments.

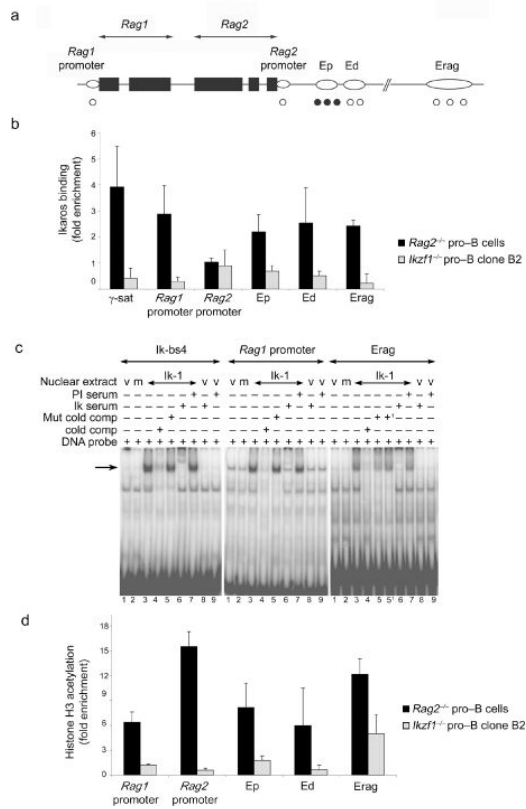


Figure 5. Ikaros binds to the *Rag* locus and regulates histone acetylation

(a) Schematic representation of the *Rag* locus. Black boxes represent *Rag1* and *Rag2* exons while open ovals represent regulatory elements. Validated (•) and putative (O) Ikaros binding sites are indicated. (b) ChIP analysis of Ikaros binding to regulatory elements of the *Rag* locus. Binding was determined in *Rag2*^{-/-} pro-B cells that express Ikaros and *Rag1*. *Ikzf1*^{-/-} pro-B cells were used as negative controls. Relative enrichment of the bound DNA over input was determined by Q-PCR after normalization to *Acta1*. Pericentromeric g-satellite sequence (g-sat) was used as a positive control for binding of Ikaros. (c) EMSAs were performed using nuclear extracts of 293T cells transfected with the Ik-1 expression vector. Control extracts were generated from 293T cells transfected with an empty vector (lane 1) or with an Ikaros-159A DNA binding mutant (lane 2). Ik-bs4 probe contains a high affinity Ikaros binding site. Other probes spanned putative Ikaros binding sites in the *Rag1* promoter and Erag enhancer. Where indicated binding reactions included competitor DNA in 50-fold molar excess or either pre-immune (PI) or anti-Ikaros antiserum (Ik serum). In lanes 5 and 5¹ two related mutant competitor DNAs were used. (d) ChIP analysis of histone H3 acetylation at regulatory elements of the *Rag* locus. Analysis was performed as described for Fig. 3b using *Actb* (encoding β-actin) for normalization. Error bars in panels (b) and (d) represent the standard deviation from the mean ($n = 3$). Data in (c) are representative of at least two independent experiments.

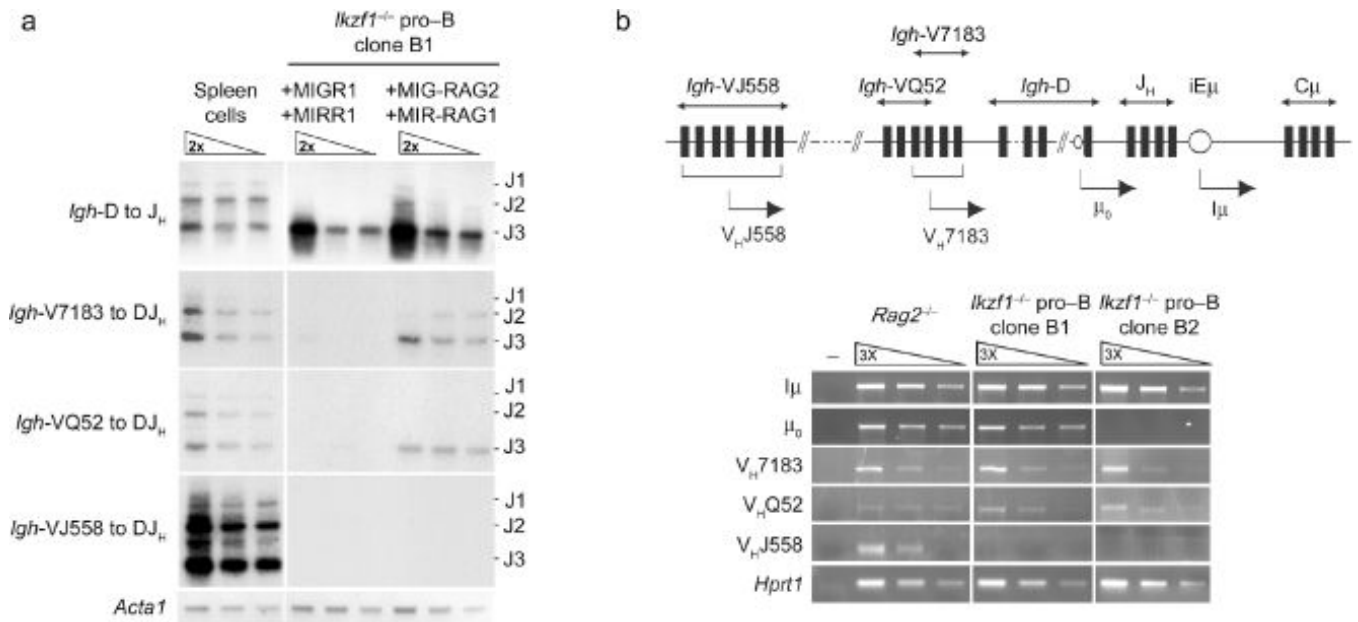


Figure 6. *Ikaros* regulates accessibility of the V_H distal segments

(a) *Ikzf1*^{-/-} pro-B cells were successively transduced with MIG-Rag2 and MIRRag1 or with MIGR1 and MIR1 as controls. Genomic DNAs from indicated cells were analyzed as in Fig. 1d, 3b to determine *Igh* rearrangement status. (b) Analysis of *Igh* germline transcripts. Top, a schematic of the *Igh* locus with the different germline transcripts denoted V_HJ558, V_HQ52, V_H7183, μ₀ and I_μ. Bottom, RT-PCR analysis of *Igh* germline transcripts in *Rag2*^{-/-} and *Ikzf1*^{-/-} pro-B cells. Serial dilutions of cDNA were analyzed after normalization to *Hprt1*. All data are representative of at least two independent experiments.

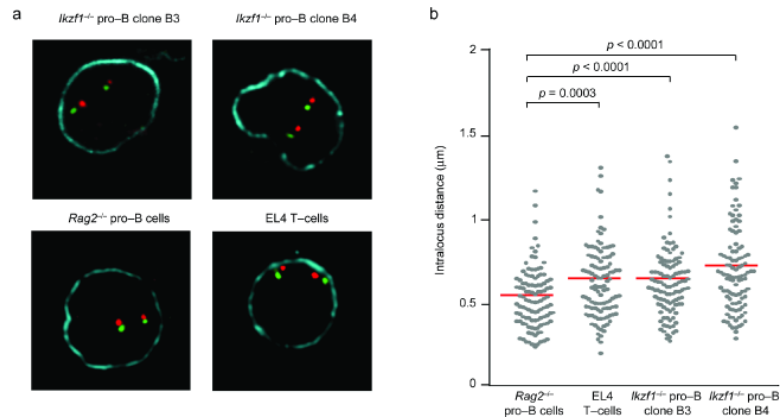


Figure 7. *Ikaros* is required for compaction of the *Igh* locus

(a) Three-dimensional immuno-FISH analysis was performed on the *Igh* locus. *Igh* alleles detected by cohybridization with probes for the V_HJ558 (red) and constant C_H (green) regions with three-dimensionally preserved nuclei of EL-4 T cells, *Rag2*^{-/-} pro-B cells and two independent *Ikzf1*^{-/-} pro-B clones. Anti-lamin B1 was used to stain the nuclear periphery (blue).

(b) Quantitative analysis of intralocus distances in the four cell types. Measurements are between V_HJ558 (red in **a**) and C_H (green in **a**) signals on the same allele. Distances represent 110 alleles per cell type. All data are representative of two independent experiments.