Ikaros is absolutely required for pre-B cell differentiation by attenuating IL-7 signals

Beate Heizmann,[1] Philippe Kastner,[1,2] and Susan Chan[1]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Institut National de la Santé et de la Recherche Médicale U964, Centre National de la Recherche Scientifique UMR7104, Université de Strasbourg, 67404 Illkirch, France
[2]Faculté de Médecine, Université de Strasbourg, 67000 Strasbourg, France

Pre-B cell receptor (pre-BCR) signaling and migration from IL-7–rich environments cooperate to drive pre-B cell differentiation via transcriptional programs that remain unclear. We show that the Ikaros transcription factor is required for the differentiation of large pre-B to small pre-B cells. Mice deleted for Ikaros in pro/pre-B cells show a complete block of differentiation at the fraction C′ stage, and Ikaros-null pre-B cells cannot differentiate upon withdrawal of IL-7 in vitro. Restoration of Ikaros function rescues pre-B cell differentiation in vitro and in vivo and depends on DNA binding. Ikaros is required for the down-regulation of the pre-BCR, Igκ germline transcription, and Ig L chain recombination. Furthermore, Ikaros antagonizes the IL-7–dependent regulation of >3,000 genes, many of which are up- or down-regulated between fractions C′ and D. Affected genes include those important for survival, metabolism, B cell signaling, and function, as well as transcriptional regulators like *Ebf1*, *Pax5*, and the *Foxo1* family. Our data thus identify Ikaros as a central regulator of IL-7 signaling and pre-B cell development.

The Journal of Experimental Medicine

CORRESPONDENCE
Susan Chan and Philippe Kastner:
scpk@igbmc.fr

Abbreviations used: 4OHT, 4-hydroxytamoxifen; B-ALL, B cell acute lymphoblastic leukemia; BCR, B cell receptor; cKO, conditional KO; HC, H chain; LC, L chain; PEC, peritoneal cavity; TAM, tamoxifen; tg, transgenic.

B cell development is marked by certain inescapable events (Pieper et al., 2013). Pro-B cells must productively rearrange the Ig H chain (HC) locus and express the surrogate λ5 and Vpre-B L chain (LC) components, as well as Igα and Igβ, to form the pre-B cell receptor (pre-BCR) complex. Pre-B cells undergo a transient proliferative phase that is dependent on pre-BCR signaling and IL-7. Continuous pre-BCR signaling and migration from IL-7–rich areas lead to cell cycle exit and germline LC transcription. Subsequent LC recombination results in BCR expression and progression to the immature B cell stage. How pre-BCR signals and loss of IL-7 cooperate to differentiate pre-B cells is a subject of intense study (Herzog et al., 2009; Corfe and Paige, 2012).

The potential role of the Ikaros transcription factor in pre-B cell differentiation has been studied in mice carrying a germline hypomorphic Ikaros mutation, which show a partial block in pro-B to pre-B cell development (Kirstetter et al., 2002). Furthermore, Ikaros represses *Igll1* (encoding λ5) transcription in transgenic (tg) mice (Sabbattini et al., 2001). Mainly, Ikaros function has been analyzed in vitro using primary pre-B cells, or pre-B cell lines, tg for IL-7 or deleted for modulators of B cell development (e.g., *Irf4*$^{-/-}$*Irf8*$^{-/-}$, *Ebf1*$^{-/-}$, *Pten*$^{-/-}$, and *Blnk*$^{-/-}$; Thompson et al., 2007; Ma et al., 2008, 2010; Reynaud et al., 2008; Alkhatib et al., 2012). These studies suggest that Ikaros induces cell cycle exit and promotes LC expression downstream of the pre-BCR. As *IKZF1* (which encodes Ikaros) deletions are frequently detected in B cell acute lymphoblastic leukemias (B-ALLs; Mullighan et al., 2008; Dupuis et al., 2013), Ikaros has been proposed to act as a tumor suppressor by cooperating with the pre-BCR to induce cell cycle arrest (Trageser et al., 2009). Recently, a greater role for Ikaros in pre-B cell development has been suggested, as Ikaros binds many genes required for BCR signaling, Ig recombination, cell growth, and proliferation (Ferreirós-Vidal et al., 2013). Nonetheless, the physiological function of Ikaros in pre-B cell differentiation remains untested.

Here we generated mice in which floxed *Ikzf1* alleles are conditionally deleted in pro/pre-B

© 2013 Heizmann et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 13 2823-2832
www.jem.org/cgi/doi/10.1084/jem.20131735

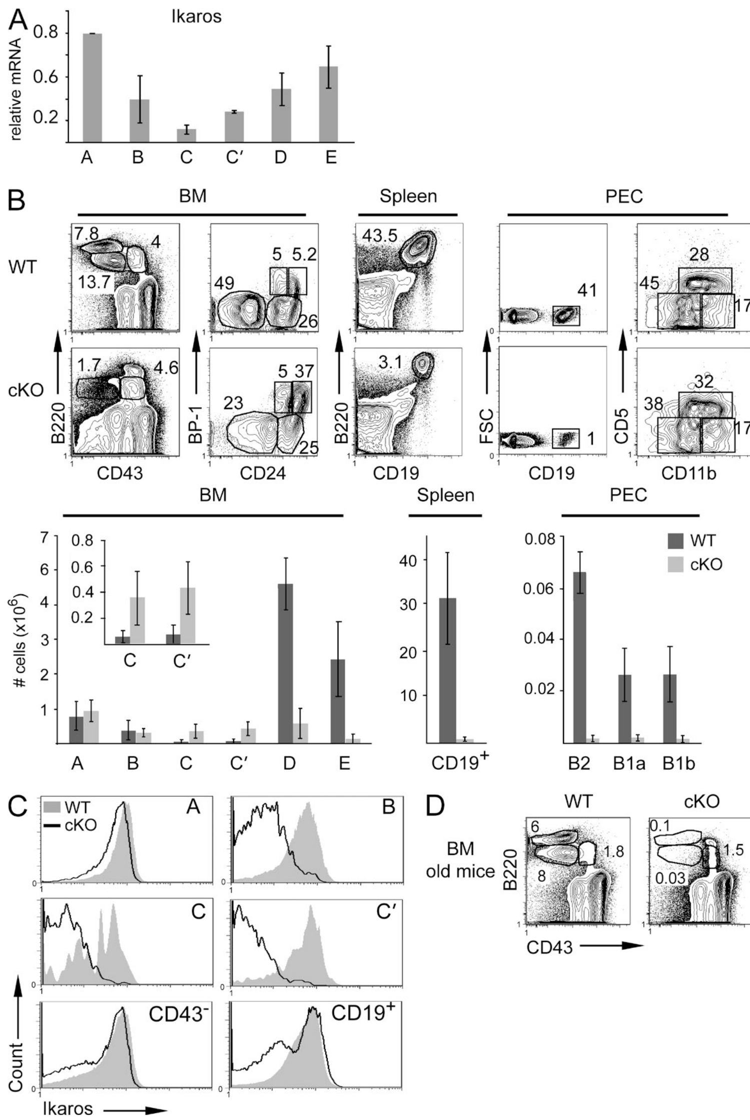

Figure 1. Pre-B cell differentiation is blocked at fraction C′ in cKO mice. (A) Analysis of Ikaros mRNA by RT-qPCR during early B cell differentiation (fractions A–E) in WT mice. Graph represents mean ± SD of two independent experiments. (B, top) B cell populations in the BM, spleen, and PEC of WT and cKO mice, as analyzed by flow cytometry. BM B220$^+$CD43$^+$ cells were further analyzed for CD24 and BP-1. Splenic B cells were analyzed for B220 and CD19. PEC CD19$^+$ cells were analyzed for CD11b and CD5 to delineate B2 (CD11b$^-$CD5$^-$), B1a (CD11b$^+$CD5$^+$), and B1b (CD11b$^+$CD5$^-$) cells. (bottom) Absolute numbers of BM, splenic, and PEC B cell populations. The inset shows fractions C and C′ on a larger scale. Graphs represent mean ± SD of three experiments, with six mice per genotype for the BM and spleen and three mice for the PEC. (C) The indicated BM and spleen cell populations were stained for intracellular Ikaros. CD43$^-$ corresponds to BM B220$^+$CD19$^+$CD43$^-$ cells. CD19$^+$ corresponds to splenic B cells. Representative of three experiments. (D) Old cKO mice (84 wk) also exhibit a block at the B220$^+$CD43$^+$ stage. Representative of seven mice per genotype in four experiments.

cells. We found that Ikaros is absolutely required for pre-B cell differentiation through a mechanism that acts primarily by attenuating the IL-7 pathway.

RESULTS AND DISCUSSION

Ikaros is absolutely required for pre-B cell differentiation

We first analyzed Ikaros expression in BM B cells. WT B220$^+$ cells were purified into fraction A (pre/pro-B; CD43$^+$CD24$^-$BP-1$^-$), B (early pro-B; CD43$^+$CD24$^+$BP-1$^-$), C (late pro-B; CD43$^+$CD24$^+$BP-1$^+$), C′ (large pre-B; CD43$^+$CD24^{hi}BP-1$^+$), D (small pre-B; CD43$^-$IgM$^-$), and E (B220$^+$IgM$^+$) cells. Ikaros mRNA levels were high in fractions A and B, reduced in C, and increased in C′, D, and E cells (Fig. 1 A). This pattern suggests an early and late role for Ikaros in B cell development.

To delete *Ikzf1* in B cell progenitors, we engineered a floxed Ikaros allele (Ik$^{f/f}$) in which exon 8 was flanked by loxP sites. Deletion by the Cre recombinase results in a null allele similar to that described by Wang et al. (1996). Ik$^{f/f}$ mice were crossed with Mb1-Cre animals, which express Cre under the control of the *Cd79a* promoter (Hobeika et al., 2006). The resulting Ik$^{f/f}$ Mb1-Cre$^+$ (conditional KO [cKO]) mice showed deletion of the Ik$^{f/f}$ allele that began in fraction A and was completed in fraction B cells; Ikaros proteins were likewise detected in fraction A but not in fraction B cells (Fig. S1). In the BM, adult cKO mice exhibited an almost complete block in B cell differentiation at the B220$^+$CD43$^+$ stage (Fig. 1 B). Furthermore, fraction C′ cells accumulated in cKO mice compared with WT (Ik$^{f/f}$ Mb1-Cre$^-$). No differences were found in intracellular μ expression and the proliferative state of WT and cKO fraction C and C′ cells (not depicted). In the periphery, B220$^+$CD19$^+$ splenic B cells, as well as peritoneal B2, B1a, and B1b cells, were almost completely lost in cKO animals.

A few B cells were observed in the cKO BM and periphery. To determine whether they had deleted the Ik$^{f/f}$ alleles,

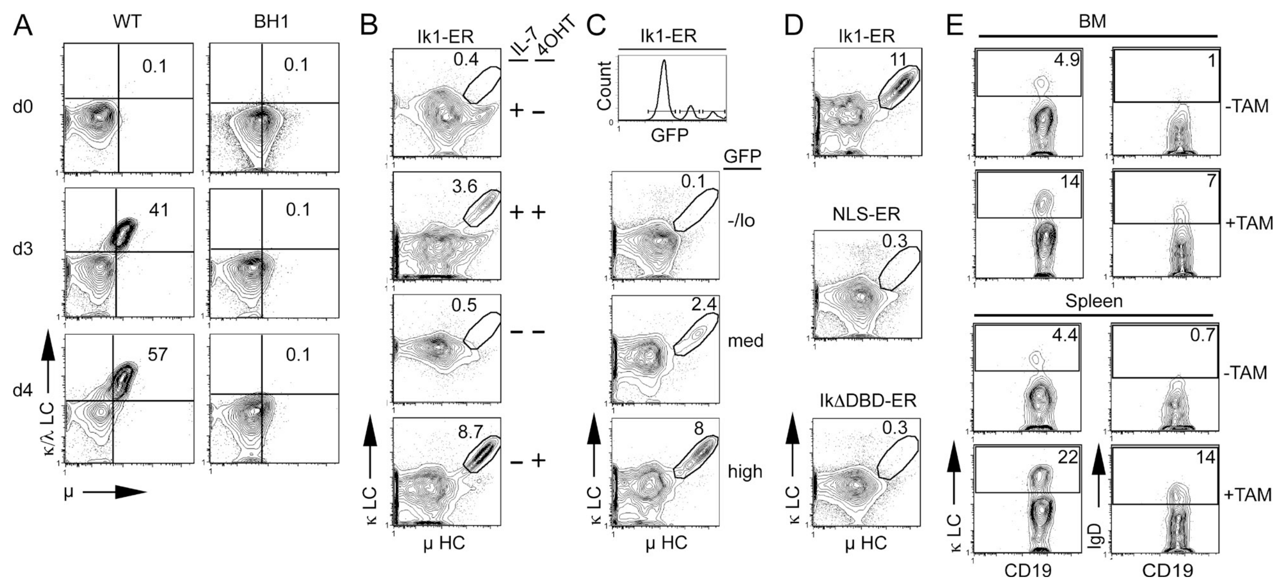

Figure 2. Ikaros rescues differentiation in vitro and in vivo. (A) WT and BH1 cultures were analyzed in the absence of IL-7. Differentiation was assessed by surface κLC and μHC. Representative of three experiments. (B) BH1–Ik1-ER–Bcl2 cells were cultured for 4 d as indicated. Differentiation was assessed as in A. (C) BH1–Ik1-ER–Bcl2 cells were cultured for 4 d with 4OHT and without IL-7. Differentiation was assessed in $GFP^{-/lo}$, GFP^{med}, and GFP^{hi} cells. (D) Differentiation was assessed in BH1–Ik1-ER–Bcl2, BH1–NLS-ER–Bcl2, and BH1–IkΔDBD-ER–Bcl2 cells cultured for 4 d with 4OHT and without IL-7. Representative of three experiments. (E) In vivo differentiation of BH1–Ik1-ER–Bcl2 cells in sublethally irradiated $Rag1^{-/-}$ mice treated with TAM or vehicle. $CD19^+$ B cells expressing κLC and IgD were monitored in the BM or spleen. Representative of three mice injected with vehicle and seven mice injected with TAM in three experiments. Unless otherwise stated, all analyzed cells were $GFP^{hi}DsRed^+$.

we evaluated them for intracellular Ikaros (Fig. 1 C). Although Ikaros was not detected in most fraction C and C′ cells, it was expressed in cKO BM $B220^+CD43^-$ cells. Furthermore, $Ikaros^{hi}$ and $Ikaros^{med}$ B cells were often detected in cKO splenic $CD19^+$ B cells, indicating that these cells had retained at least one functional *Ikzf1* allele, probably as the result of incomplete deletion. We therefore conclude that Ikaros is absolutely required for B cell differentiation from the fraction C′ stage.

$Ik^{f/f}$ $Mb1$-Cre^+ mice do not develop leukemias

As *IKZF1* deletions are associated with human B-ALL, we asked whether Ikaros deficiency in B cells leads to the development of B cell leukemias. cKO (*n* = 7) and WT (*n* = 7) mice were followed over 18 mo. Old cKO mice showed a similar block at the pre-B cell stage but were otherwise healthy (Fig. 1 D). No apparent signs of leukemia were detected (not depicted). Thus, Ikaros deletion in fraction B cells does not initiate transformation.

B cell differentiation is rescued by Ikaros expression in vitro and in vivo

To determine whether Ikaros reexpression rescues pre-B cell development, we set up an in vitro gain-of-function system using cKO and WT BM cells. As pro/pre-B cells can be expanded in IL-7 and induced to differentiate upon IL-7 withdrawal, multiple IL-7–dependent cultures were set up for both genotypes. WT cultures were $B220^+CD43^+CD24^+BP\text{-}1^{-/+}$; they resembled fraction B and C cells and expressed λ5 but not surface μ (Fig. S2 A). Very few fraction C′ cells were present, which mimicked the in vivo phenotype. These cultures grew poorly and could not be maintained over time (not depicted). In contrast, cKO cultures grew robustly and were easily maintained as cell lines (BH1-3). The results presented here were obtained with BH1 cells; they were reproducibly repeated with BH2 and BH3 cells. BH1 cells showed deletion of the $Ik^{f/f}$ allele and lacked Ikaros proteins (Fig. S2, B and C). They resembled fraction C′ cells ($B220^+CD43^+CD24^{hi}BP\text{-}1^+$, $λ5^+$, surface $μ^+$; Fig. S2 A). Stimulation with anti-μ antibodies induced an intracellular Ca^{2+} response (Fig. S2 D), indicating the presence of a functional pre-BCR. Conversely, WT cultures did not respond to anti-μ, although they displayed a Ca^{2+} flux to ionomycin. BH1 cultures therefore recapitulated the in vivo accumulation of fraction C′ cells in cKO animals. BH1 and WT cultures were subjected to IL-7 withdrawal, and the expression of κ/λLC and μHC was monitored (Fig. 2 A). Although WT cultures differentiated into BCR^+ cells, BH1 cells did not, indicating that Ikaros deficiency blocks pre-B cell differentiation in vitro.

BH1 cells were transduced to express the Ikaros-1 isoform fused to the ligand-binding domain of the estrogen receptor (Ik1-ER) in conjunction with GFP. Ik1-ER translocates from the cytoplasm to the nucleus upon 4-hydroxytamoxifen (4OHT) treatment. However, GFP^+ BH1–Ik1-ER cells were very sensitive to Ikaros activation and died within 24 h, even in the presence of IL-7, although they expressed similar levels

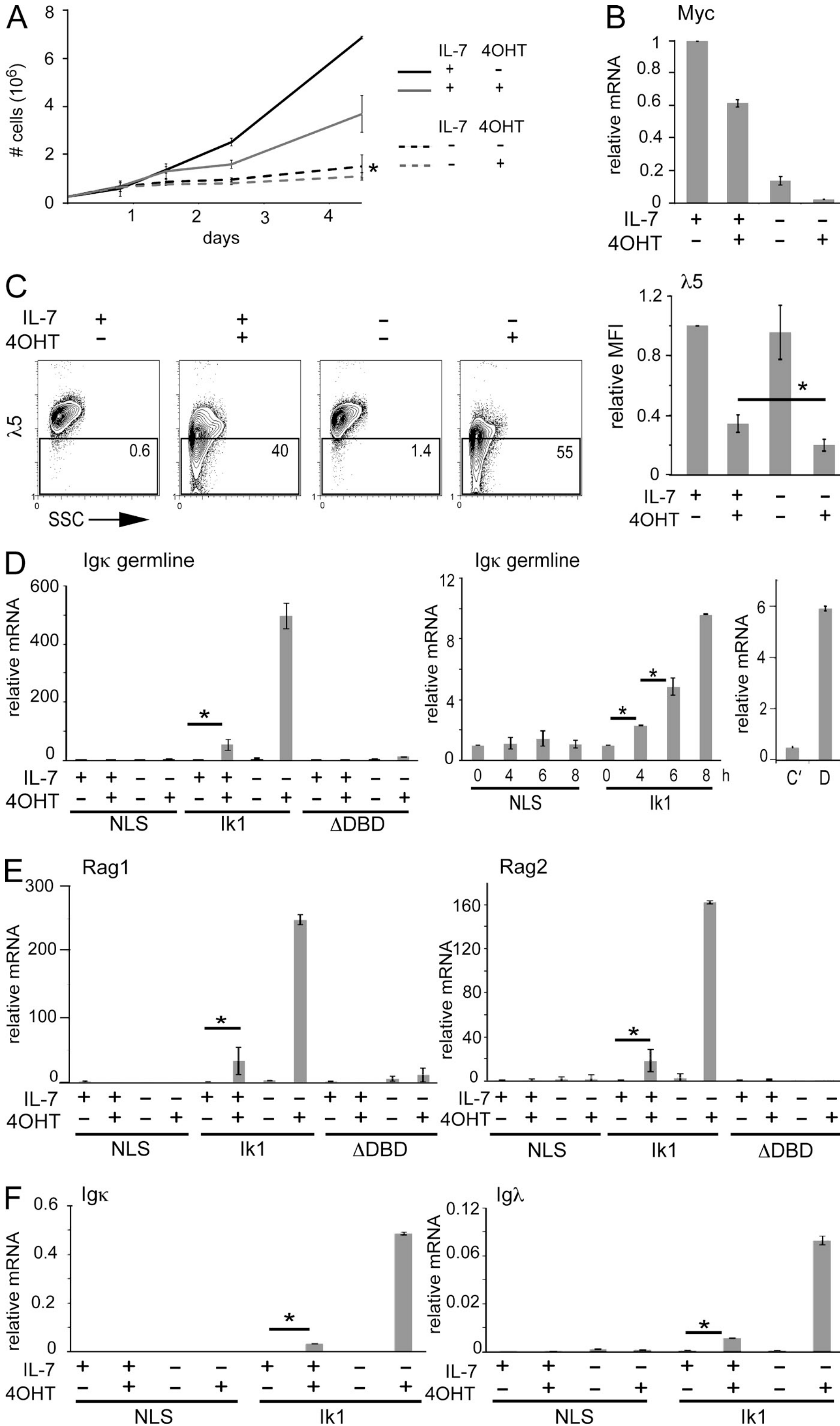

Figure 3. Ikaros represses Myc and λ5 expression and induces LC transcription and rearrangement. (A) Proliferation of BH1–Ik1-ER–Bcl2 cells cultured as indicated. Graph represents mean ± SD of three experiments. Significance for all panels was calculated using the unpaired Student's *t* test. *, $P < 0.01$. (B) *Myc* mRNA expression as measured by RT-qPCR in BH1–Ik1-ER–Bcl2 cells cultured for 24 h, as indicated. Graph represents mean ± SD of duplicate samples from one of two experiments. (C) Analysis of intracellular λ5 in BH1–Ik1-ER–Bcl2 cells cultured for 24 h, as indicated. Graph represents mean ± SD of the mean fluorescence intensity (MFI) for λ5 from three experiments. Values were calculated relative to that of the sample cultured with IL-7 and without 4OHT, which was set to 1. *, $P < 0.01$. (D, left) RT-qPCR analysis of Igκ germline transcription in BH1–Ik1-ER–Bcl2 (Ik1), BH1–NLS-ER–Bcl2 (NLS), and BH1–IkΔDBD-ER–Bcl2 (ΔDBD) cells cultured for 24 h, as indicated. Graph represents mean ± SD of three experiments. (middle) Igκ germline transcription in BH1–Ik1-ER–Bcl2 and BH1–NLS-ER–Bcl2 cells cultured in the presence of IL-7 and 4OHT for the indicated times. Graph represents mean ± SD of duplicate samples from one of two experiments. (right) Igκ germline transcription in WT fraction C′ and D cells. Graph represents mean ± SD of duplicate samples from one experiment. *, $P < 0.01$. (E) Rag1 and Rag2 mRNA expression in the indicated cell lines cultured as indicated for 24 h. Graphs represent mean ± SD of two experiments. *, $P < 0.01$. (F) Analysis of Igκ and Igλ rearrangements in the indicated samples cultured for 24 h. Graphs represent mean ± SD of two experiments. *, $P < 0.01$. All analyzed cells were $GFP^{hi}DsRed^{+}$.

of Ikaros mRNA compared with WT fraction C′ and D cells (not depicted). We therefore transduced BH1–Ik1-ER cells to express the antiapoptotic protein BCL-2 and a DsRed reporter, which efficiently prevented apoptosis in 4OHT-treated $GFP^{+}DsRed^{+}$ BH1–Ik1-ER–Bcl2 cells (not depicted). As negative controls, BH1–NLS-ER–Bcl2 cells, which expressed a nuclear translocation signal sequence fused to ER (to control for nonspecific ER- and Bcl-2–related effects), and BH1–IkΔDBD-ER–Bcl2 cells, which expressed Ik1 without the DNA binding domain, were generated. Substantial Ik1-ER

and IkΔDBD-ER proteins were translocated to the nucleus upon 4OHT treatment, indicating that these proteins were stable and functional (Fig. S2 E). When BH1–Ik1-ER–Bcl2 cells were subjected to IL-7 withdrawal and 4OHT, pre-B cell differentiation was rescued, as measured by surface μ and κ/λLC expression (Fig. 2 B). Interestingly, Ik1 expression also induced differentiation in the presence of IL-7, although to a lesser degree. This effect was dose dependent, as GFP^{hi} cells showed more $\mu^+\kappa^+$ cells than GFP^{med} cells (Fig. 2 C). No differentiation was observed in NLS-ER or IkΔDBD-ER cells (Fig. 2 D), demonstrating the importance of DNA binding for Ikaros function.

To determine whether Ikaros rescues pre-B cell differentiation in vivo, BH1–Ik1-ER–Bcl2 cells were adoptively transferred into $Rag1^{-/-}$ mice. These mice were treated with tamoxifen (TAM) for 5 d and evaluated 2 d later (Fig. 2 E). Significantly more B cells expressing κLC and IgD were detected in the BM and spleens of TAM- versus vehicle-treated mice. The small number of differentiated B cells in the control mice was puzzling and may reflect an in vivo leakiness of the Ik1-ER or improved pre-B cell survival because of the BCL2 protein. These results demonstrate that Ikaros alone is sufficient to induce pre-B cell differentiation in vitro and in vivo.

Ikaros cooperates with IL-7 withdrawal for cell cycle exit, germline LC transcription, and LC rearrangement

We first analyzed the impact of Ikaros on proliferation. Ikaros activation in the presence of IL-7 reduced the proliferation of BH1–Ik1-ER–Bcl2 cells (Fig. 3 A), an effect not seen in BH1–IkΔDBD-ER–Bcl2 cells (not depicted). As expected, IL-7 withdrawal alone led to an almost complete proliferation arrest. The combination of Ikaros and IL-7 withdrawal further reduced proliferation in a small but significant manner. The reduction in proliferation was caused by a decrease in cycling cells and not an increase in cell death (not depicted). Similar results were observed with *Myc* expression. Both Ikaros and IL-7 withdrawal alone reduced *Myc* mRNA levels, and this effect was cumulative (Fig. 3 B). Thus Ikaros and IL-7 withdrawal cooperate to promote cell cycle exit.

We then evaluated pre-BCR expression, Ig LC germline transcription, and LC rearrangement. 4OHT-treated BH1–Ik1-ER–Bcl2 cells decreased λ5 expression (Fig. 3 C), indicating loss of pre-BCR expression. This was Ikaros specific as it was not seen with IL-7 withdrawal alone. However, the combination of Ikaros and IL-7 withdrawal enhanced λ5 down-regulation. Strikingly, Ikaros induced the appearance of Igκ germline transcripts as early as 4 h after 4OHT treatment (Fig. 3 D); at 6 h, this expression reached that of fraction D cells. Igκ germline transcripts were detected in the presence of IL-7 and increased upon IL-7 withdrawal. IL-7 withdrawal had no effect on its own without Ikaros. We then asked whether Ikaros affects LC rearrangement. In the absence of Ikaros, BH1–Ik1-ER–Bcl2 cells did not express detectable *Rag1,2* mRNA (Fig. 3 E). However, Ikaros induced *Rag1,2* mRNA levels in the presence of IL-7, which was enhanced more than fivefold upon IL-7 withdrawal. In addition, Ikaros

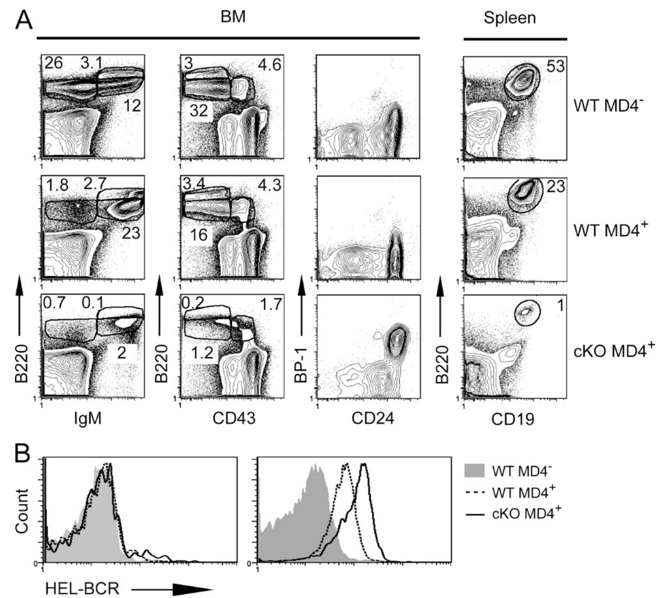

Figure 4. Tg BCR expression does not rescue differentiation. (A) BM and splenic B cell populations of the indicated mice. BM $B220^+CD43^+$ cells were further analyzed for BP-1 and CD24. Representative of three experiments. (B) HEL BCR expression in fraction A ($B220^+CD43^+CD19^-$; left) and B–C′ ($B220^+CD43^+CD19^+$; right) cells. Note that the higher level of HEL BCR on cKO $MD4^+$ compared with WT $MD4^+$ cells likely reflects the accumulation of fraction C′ cells in the cKO animals. In this figure, WT denotes $Ik^{+/f}$ $Mb1\text{-}Cre^-$.

alone induced κ/λLC rearrangement, and IL-7 withdrawal further enhanced rearranged κ/λLC mRNA levels >10-fold (Fig. 3 F). Thus, Ikaros cooperates with IL-7 withdrawal to allow cell cycle exit, pre-BCR down-regulation, and LC expression.

To determine whether a functional BCR is sufficient to overcome the loss of Ikaros, we crossed cKO mice with MD4 tg animals, which express a BCR specific for hen egg lysozyme (Goodnow et al., 1988). cKO $MD4^+$ cells remained blocked at fraction C′ (Fig. 4 A), and their surface marker expression was similar to that of cKO $MD4^-$ cells, even though the tg BCR was expressed at the proper fraction B and C′ stages (Fig. 4 B). Thus, Ikaros is required for other events in addition to promoting BCR expression.

Ikaros is mainly an activator of gene expression in pre-B cells

To gain insight into the gene expression changes induced by Ikaros, we analyzed the effects of Ikaros reexpression in BH1–Ik1-ER–Bcl2 cells at 6 and 24 h by transcriptome profiling, in the presence of IL-7 to focus on Ikaros-associated events. 4OHT treatment for 24 h resulted in the activation and repression (>1.4-fold in both experiments) of 536 and 399 genes, respectively (Fig. 5 A). Most genes were already altered after 6 h (Fig. 5 B), including most of the down-regulated (363/399 genes or 91%; cluster 2) and half of the up-regulated genes (285/536 genes or 53%; cluster 1). We then compared

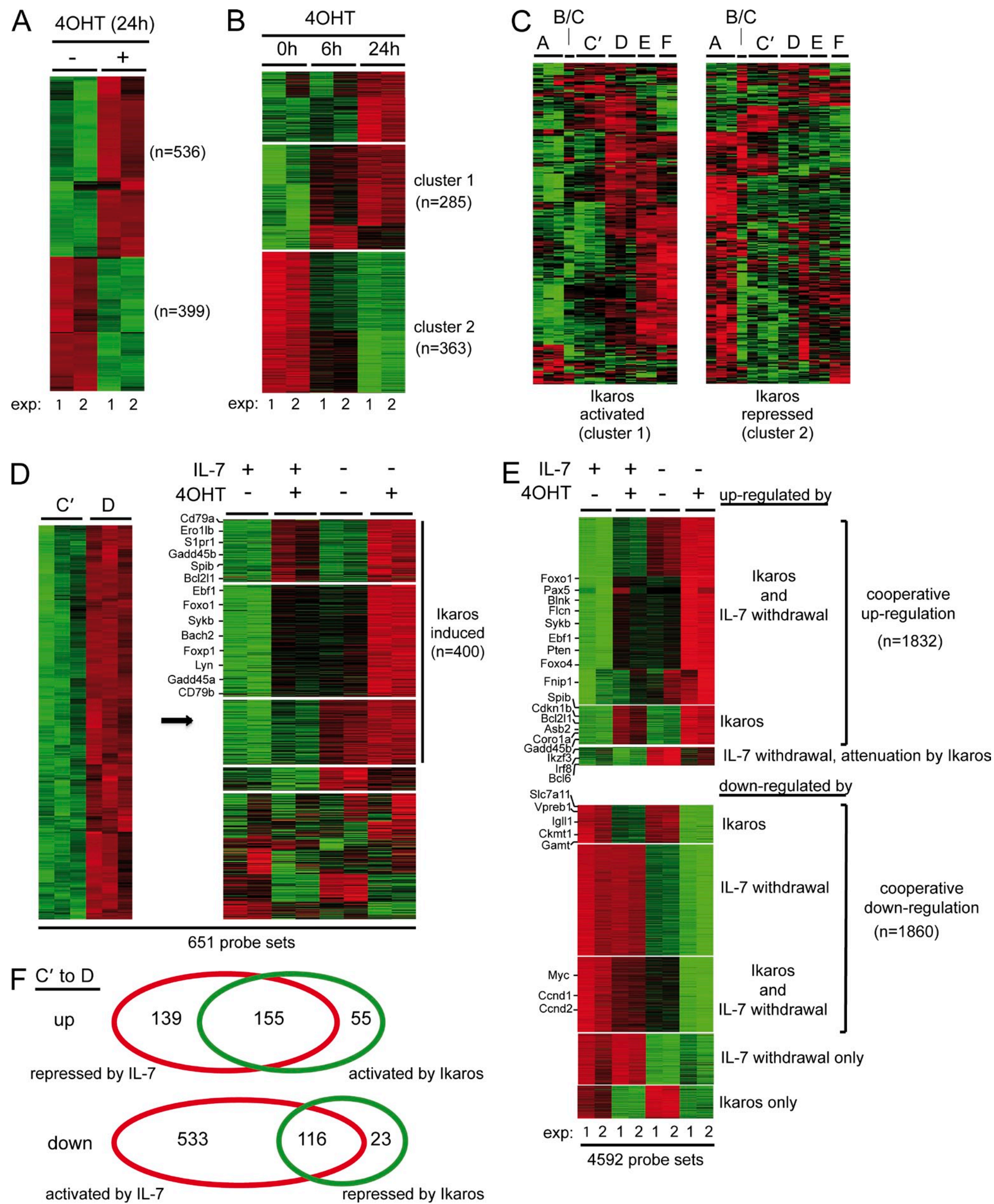

Figure 5. Transcriptome analysis of Ikaros-regulated genes in pre-B cells. (A) BH1–Ik1-ER–Bcl2 cells were cultured for 24 h as indicated in two experiments (exp. 1 or 2). Probe sets corresponding to known genes, and differentially expressed in each experiment, were selected (difference in RMA expression values >0.5 up or down) and visualized by hierarchical clustering. Red, up-regulated; green, down-regulated. With these criteria, no probe set displayed opposite regulation patterns between experiments, indicating a false discovery rate of ~0. (B) Probe sets selected in panel A were visualized by K-means clustering after 6 and 24 h of 4OHT. (C) ImmGen datasets corresponding to WT BM B cell subsets (GEO accession no. GSE15907; only one fraction of the B/C sample was used, as the other appeared contaminated). Probe sets corresponding to cluster 1 or cluster 2 from panel B were selected, and their expression was visualized by hierarchical clustering. (D) Probe sets up-regulated between fractions C′ and D in the ImmGen dataset were selected (difference in RMA > 0.6) and visualized by hierarchical clustering (left). Their expression was then clustered by K-means clustering with the data from BH1–Ik1-ER–Bcl2 cells, as indicated. (E) Probe sets were selected as up- or down-regulated in BH1–Ik1-ER–Bcl2 cells upon Ik1 expression or IL-7 deprivation, or both conditions together, relative to control cultures (with IL-7, no 4OHT), where the differences in RMA were >0.5, up or down. Clusters of similarly regulated probe sets were identified by K-means clustering. The positions of specific genes are indicated. (F) Venn diagrams showing the overlap between genes that are up- or down-regulated by Ikaros and IL-7 withdrawal, among genes modulated between fractions C′ and D. (top) Genes up-regulated between fractions C′ and D were selected as in panel D. The genes were compared with genes activated by IL-7 withdrawal, Ikaros, or both conditions together (selected as in panel E). Genes were considered regulated by Ikaros when they were either regulated by 4OHT or the combination of 4OHT and IL-7 withdrawal, but not IL-7 withdrawal alone. Only genes that were regulated by Ikaros and/or IL-7 are shown. (bottom) Similar representation for genes down-regulated between fractions C′ and D. Note that the apparent discrepancy between the numbers of regulated genes in this panel and other panels originates from the different criteria used for gene selection.

our data with the published genome-wide Ikaros binding profiles in primary pre-B cells to identify direct Ikaros target genes (Ferreirós-Vidal et al., 2013). Ikaros binding was found in >80% of the genes up- or down-regulated at both 6 and 24 h, suggesting that many of the Ikaros-regulated genes may be direct targets (see Fig. S3 for representative genes).

To determine whether the genes regulated by Ikaros in BH1 cells correspond to genes normally regulated during pre-B cell differentiation, we compared our data with those from the ImmGen Consortium for fractions A–F (Fig. 5 C). Comparisons of genes changed after 6 h of 4OHT (clusters 1 and 2) revealed that most of the Ikaros-activated genes were up-regulated in fractions C′ and D (190 genes; left), whereas those repressed by Ikaros were down-regulated in fractions B/C (234 genes), C′ (24 genes), and D (54 genes). Conversely, when we compared the genes up-regulated between fractions C′ and D (651 genes with >1.5-fold change) with those activated by Ikaros in BH1 cells (Fig. 5 D), 400 genes (60%) were common to both. In contrast, only 76 genes (9%) down-regulated between fractions C′ and D were repressed by Ikaros in BH1 cells (not depicted). These results suggest that Ikaros functions mainly as an activator in fraction C′.

Ikaros cooperates with IL-7 withdrawal to establish a transcriptional program

Our results suggest that Ikaros cooperates with IL-7 withdrawal for multiple events. We therefore evaluated the gene expression changes in BH1–Ik1-ER–Bcl2 cells in response to Ikaros and/or IL-7 withdrawal (Fig. 5 E). 4,592 genes were up- or down-regulated >1.4-fold between conditions in two independent experiments. Of these, Ikaros cooperated with IL-7 withdrawal to up-regulate the expression of 1,832 genes. Most (n = 1,411) were separately induced by Ikaros or IL-7 withdrawal, and their expression was further increased under both conditions. They included genes important for pre-BCR and BCR signaling (e.g., *Blnk*, *Syk*, *Lyn*, *Btk*, and *Pten*) and B cell regulators like *Pax5*, *Foxo1*, and *Ebf1*. A second group (n = 421) was induced only by Ikaros, and their expression increased upon IL-7 withdrawal (e.g., *Cdkn1b*, *Spib*, and *Bcl2l1*). In contrast, a smaller group was induced only by IL-7 withdrawal, but was less activated upon Ikaros reexpression (e.g., *Ikzf3*, *Irf8*, and *Bcl6*).

Ikaros also cooperated with IL-7 withdrawal to down-regulate 1,860 genes. These genes were primarily down-regulated by either Ikaros or loss of IL-7 or similarly affected by either condition. In all cases, the combination of Ikaros and IL-7 withdrawal led to a cumulative down-regulation of gene expression. Affected genes included *Myc* and genes implicated in cell growth and proliferation (i.e., tRNA biosynthesis, nucleotide biosynthesis, glycolysis, mitochondrial function, and DNA replication), which are broadly influenced by MYC (Eilers and Eisenman, 2008). Only a few genes were singularly regulated by Ikaros or IL-7 withdrawal and not further affected by the combination of both (Fig. 5 E, bottom).

Importantly, Ikaros activated 155/210, and repressed 116/139, genes between fractions C′ and D that were similarly regulated by IL-7 withdrawal. This corresponds to 75% of the genes regulated by Ikaros at these stages (Fig. 5 F). These data highlight a strong synergy between Ikaros and IL-7 withdrawal during pre-B cell differentiation.

Conclusions

Our results demonstrate that Ikaros is a major, nonredundant, regulator of B cell development. The Ikaros mutant phenotype closely resembles that of *Irf4,8*$^{-/-}$ double mutant mice, which also show a block at the fraction C′ stage (Lu et al., 2003), strongly suggesting that Ikaros and IRF4,8 share a common pathway. Interestingly, *Irf4,8*$^{-/-}$ pre-B cells do not express Ikaros, and either IRF4 or IRF8 can activate Ikaros transcription (Ma et al., 2008). Thus, Ikaros may be a critical downstream target of IRF4,8 in pre-B cells.

Ikaros is required for pre-BCR down-regulation and Ig LC expression. In addition to its known effect on λ5 repression (Sabbattini et al., 2001; Thompson et al., 2007; Ma et al., 2008), Ikaros rapidly activates Igκ germline transcription. Interestingly, Ikaros binds the Ig Eκ3′ and Eκi enhancers in pre-B cells (Fig. S3). Ikaros appears to override the repressive effect of IL-7–STAT5 on Igκ transcription (Malin et al., 2010; Mandal et al., 2011), as Igκ transcription is induced by Ikaros in the presence of IL-7, and Ikaros is required for Igκ transcription upon IL-7 withdrawal. Ikaros also activates *Rag1,2* transcription to promote LC recombination and binds the Erag enhancer and both *Rag1,2* promoters (Fig. S3). As Ikaros also induces the expression of *Foxp1*, *Foxo1*, and *Foxo4*, as well as *Gadd45a* and *Pten* (Fig. 5, D and E), which promote FOXO function (Amin and Schlissel, 2008; Herzog et al., 2008), Ikaros may increase the expression of critical regulators of *Rag1,2* transcription in a feed forward mechanism.

These events are essential for BCR expression but not sufficient for differentiation. We found that Ikaros regulates a large cohort of fraction C′ genes. These include genes involved in lymphocyte trafficking (*S1pr1*), actin filament organization, and cell migration (*Coro1a* and *Asb2*), suggesting that Ikaros may influence pre-B cell migration from IL-7–rich pockets. Ikaros up-regulates the expression of the genes encoding folliculin (*Flcn*) and folliculin-interacting protein 1 (*Fnip1*), important for energy homeostasis, which were recently identified as essential checkpoints in pre-B cells (Baba et al., 2012; Park et al., 2012). Ikaros also down-regulates genes involved in the mitochondrial creatine pathway (*ckmt1* and *gamt*), implicated in ATP synthesis. Furthermore, Ikaros affects genes linked to redox homeostasis, like *Slc7a11* (repressed) or *Ero1b* and *Mgst1* (activated). Lastly, Ikaros activates the expression of prosurvival genes like *Bcl2l1*, which encodes BCL-xL, and *Gadd45a,b* (Fig. 5, D and E; Engelmann et al., 2008) physiologically induced in fraction C′ cells. As BCL-xL is an essential survival factor in pre-B cells (Motoyama et al., 1995), its low expression in cKO pre-B cells may explain why the abrupt reintroduction of Ikaros induces cell death in BH1 cells. Thus, Ikaros is important for the regulation of many developmentally regulated genes, but future studies will be needed to distinguish direct from indirect targets.

An important question is how Ikaros is regulated during pre-B cell differentiation. Ikaros transcripts and protein are down-regulated in fraction C and up-regulated in fraction C′ cells, suggesting that Ikaros function may dip when the pre-BCR is first expressed, and that pre-BCR signaling may up-regulate Ikaros expression. Furthermore, FOXO1 may regulate Ikaros through correct mRNA splicing (Alkhatib et al., 2012), and SYK may influence Ikaros localization and binding activity (Uckun et al., 2012). Additional research will be needed to determine how Ikaros activity is modulated during differentiation.

Finally, our results suggest that Ikaros deficiency may contribute to B-ALL development by antagonizing STAT5 function. Indeed, *IKZF1* mutations are strongly associated with events leading to STAT5 activation (BCR-ABL expression or *CRLF2* amplifications; Mullighan et al., 2008; Harvey et al., 2010). *IKZF1* haploinsufficiency may enhance STAT5 signaling in tumor cells where STAT activity is already up-regulated. This may explain why *IKZF1* deletions are frequently secondary hits in B-ALL (Kastner et al., 2013) and why $Ik^{f/f}$ $Mb1\text{-}Cre^{+}$ mice do not develop disease.

The key to Ikaros function might thus lie in its ability to turn off the IL-7–dependent gene expression program. As IL-7 functions through STAT5A,B, Ikaros may target the machinery that activates STAT5 or interact with essential STAT5-responsive genes to modulate their transcription. Interestingly, only a small fraction of Ikaros-regulated genes appear to be critical for its function in early B cell development and as a tumor suppressor (Schjerven et al., 2013). These mechanisms will be important to elucidate in the future.

MATERIALS AND METHODS

Mice. The $Ik^{f/f}$ mouse line was engineered by inserting loxP sites around exon 8 via homologous recombination in ES cells (performed at the Institut Clinique de la Souris), similar to the mutation described by Wang et al. (1996). To generate $Ik^{f/f}$ $Mb1\text{-}Cre^{+}$ mice, $Ik^{f/f}$ mice were bred with Mb1-Cre tg mice (gift of M. Reth, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany; Hobeika et al., 2006) and progeny were backcrossed to obtain $Ik^{f/f}$ $Mb1\text{-}Cre^{+}$ (cKO) and control mice ($Ik^{f/f}$ $Mb1\text{-}Cre^{-}$). The MD4 and $Rag\text{-}1^{-/-}$ mouse lines were obtained from the Jackson Laboratory ($Tg(IghelMD_4)_4Ccg$ and $Rag1^{tm1Mom}$ strains). All animal experiments were approved by the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) ethical committee (Com'Eth #2012-092).

Cell lines, cell culture, transfection, and retroviral transduction. The cKO and WT pro/pre-B cell lines were established from BM cells from $Ik^{f/f}$ $Mb1\text{-}Cre^{+}$ or $Ik^{f/f}$ $Mb1\text{-}Cre^{-}$ mice, respectively. These lines were cultured in Iscove's medium supplemented with 10% FCS, 100 U/ml penicillin-streptomycin, 2 mM glutamine, 50 µM 2-mercaptoethanol, and 8% conditioned medium from a J558L cell line stably overexpressing mouse IL-7 (gift of M. Reth; Winkler et al., 1995). Retroviruses (pMCSV-mBcl2-DsRed [gift of J. Ghysdael, Institut Curie, Orsay, France; Gachet et al., 2013], pMIG-Ik1-ER [generated by A.S. Geimer Le Lay, IGBMC, Illkirch, France], pMIG-NLS-ER, and pMIG-Ik1ΔDBD-ER [lacking Ikaros amino acids 119–223; generated by A. Apostolov, IGBMC]) were generated by $CaCl_2$ transfection of the EcoPhoenix cell line (gift of G. Nolan, Stanford University, Stanford, CA). The Mig vector was a gift of W. Pear (University of Pennsylvania, Philadelphia, PA). Retrovirus-containing supernatants were harvested after 36 h. Viral infections of 2×10^5 BH1 cells were performed by the addition of 500 µl supernatant followed by 3-h centrifugation at 2,000 rpm. Cells were expanded and sorted based on the expression of GFP and DsRed.

Antibodies, flow cytometry analysis, and cell sorting. Cells from total BM, spleen, or peritoneal cavity (PEC) were analyzed (LSRII; BD) or purified (FACSAria SORP; BD) as fraction A ($B220^{+}CD19^{-}IgM^{-}CD43^{+}CD24^{-}BP\text{-}1^{-}$), B ($B220^{+}CD19^{+}IgM^{-}CD43^{+}CD24^{+}BP\text{-}1^{-}$), C ($B220^{+}CD19^{+}IgM^{-}CD43^{+}CD24^{+}BP\text{-}1^{+}$), C′ ($B220^{+}CD19^{+}IgM^{-}CD43^{+}CD24^{hi}BP\text{-}1^{+}$), D ($B220^{+}CD19^{+}IgM^{-}CD43^{-}$), E ($B220^{+}CD19^{+}IgM^{+}CD43^{-}$), splenic ($B220^{+}CD19^{+}$), PEC B2 ($CD19^{+}CD11b^{-}CD5^{-}$), B1a ($CD19^{+}CD11b^{+}CD5^{+}$), and B1b ($CD19^{+}CD11b^{+}CD5^{-}$) B cells. Purity was >98%. Help for cell sorting was provided by C. Ebel (IGBMC). The following antibodies or reagents were used: anti–B220-PECy7 (BioLegend), anti–CD43 (S7)-PE, anti–BP-1–FITC, anti–CD24-bio, anti–λ5 (LM34)-bio, anti–Igκ-bio, anti–CD19-PerCPCy5.5 (BD), anti–IgD (11-26c)-eFluor450N, anti–CD5 (53-7.3)-bio, anti–CD11b (M1/70)-APC (eBioscience), anti–mouse IgG-Cy5, anti–IgM-Cy5 (Jackson ImmunoResearch Laboratories, Inc.), anti–Igλ-bio (SouthernBiotech), streptavidin–Alexa Fluor 405 (Invitrogen), anti-Ikaros (A3; in-house rabbit polyclonal antibody against the Ikaros C terminus), and anti-HEL (gift of A.S. Korganow, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France). Intracellular staining was performed according to the Foxp3 staining protocol (eBioscience). Dead cells were excluded with 10 µg/ml propidium iodide. Cell cycle analysis was performed with DAPI. For staining of the HEL-specific BCR, cells were saturated with 200 ng/ml HEL, followed by incubation with the anti-HEL antibody. Data were analyzed with FlowJo software (Tree Star).

In vitro and in vivo differentiation assays. For in vitro differentiation, 10^6 cells were plated in Iscove's medium supplemented or not with 8% IL-7 or 100 nM 4OHT (Sigma-Aldrich). For in vivo differentiation, sublethally irradiated (5 Gy) $Rag\text{-}1^{-/-}$ mice were injected i.v. with 2×10^7 cells. From day 2 after transplantation, mice were injected daily i.p. with 500 µg TAM (Sigma-Aldrich) in sunflower oil for 5 d, and B cells were assessed 2 d after the last injection.

Primers, analysis of VDJ recombination analysis, and RT-qPCR. Genomic PCR amplification of Ikaros was performed with the following primers: *Ikzf1* flox, 5′-GAGGACCAGATATAAGGCAGCTGG-3′ and 5′-GGCCATCAACGGCATGGAAACGATAA-3′; and *Ikzf1* deletion, 5′-AGCACAGGTTGGACAATACCTGAAA-3′ and 5′-GGCCATCAACGGCATGGAAACGATAA-3′.

For RT-qPCR, RNA was extracted using RNeasy kit (QIAGEN). 350 ng RNA was reverse transcribed with Superscript Reverse Transcription (Invitrogen). RT-qPCR was performed with the SYBR Green PCR Master Mix (Sigma-Aldrich, Roche, or QIAGEN) on an LC480 light cycler (Roche). Oligonucleotides for Igκ germline transcript and rearranged Igκ and Igλ were used as described previously (Fuxa et al., 2004; Mandal et al., 2011).

Other primers used were as follows: *Hprt*, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and 5′-GATTCAACTTGCGCTCATCTTAGGC-3′; *Rag1*, QT00243621 (QIAGEN); *Rag2*, QT00253414 (QIAGEN); *Ikzf1*, QT01060689 (QIAGEN); and *Myc*, Mm.PT.56.28494642 (Integrated DNA Technologies).

Measurement of calcium release. 3×10^6 cells were incubated with 5 µg/ml Indo-1 (Invitrogen) and 0.5 µg/ml Pluronic acid (Invitrogen) in Iscove's medium containing 1% FCS for 45 min at 37°C. The cells were resuspended in Iscove's medium containing 1% FCS, and the Ca^{2+} response was induced by addition of 20 µg/ml anti-µ (Jackson ImmunoResearch Laboratories, Inc.). As a positive control, cells were stimulated with 1 µM ionomycin.

Microarray and transcriptome analysis. 10^7 cells were treated for 6 or 24 h with or without 4OHT in the presence or absence of IL-7, as described above. RNA was extracted with the RNeasy kit. Biotinylated single-strand cDNA targets were prepared, starting from 150 ng of total RNA, using the

Ambion WT Expression kit and the Affymetrix GeneChip WT Terminal Labeling kit. After fragmentation and end-labeling, 1.9 µg cDNA was hybridized for 16 h at 45°C on GeneChip Mouse Gene 1.0 ST arrays (Affymetrix). Data were normalized with the Robust Multi-array Average (RMA) software using default settings and analyzed with Excel (Microsoft) and Cluster 3. Probe sets that did not correspond to an identified gene were not considered for analysis. Clusters were visualized with Treeview. Help with analyses were provided by C. Thibault, D. Dembélé, and A. Oravecz (IGBMC). The data are available in the Gene Expression Omnibus database under accession no. GSE51350.

Western blot. Cytoplasmic and nuclear extracts from 10^7 cells were prepared using cytoplasmic lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and protease inhibitor cocktail) and nuclear lysis buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 25% glycerol, and protease inhibitor cocktail), respectively. Lysates were separated by SDS-PAGE on a 10% gel, and Western blotting was performed using an enhanced chemiluminescence system. The following antibodies were used for detection: anti-Ikaros, anti-ER, anti-TBP (all in-house), and anti–β-actin (BD).

Online supplemental material. Fig. S1 shows an analysis of Ikaros expression in cKO mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131735/DC1.

We thank M.C. Birling, M. Reth, J. Ghysdael, A.S. Korganow, W. Pear, G. Nolan, A.S. Geimer Le Lay, A. Apostolov, A. Oravecz, C. Thibault, D. Dembélé, P. Marchal, M. Gendron, S. Falcone, and C. Ebel for reagents, help, and discussion.

We received grants from Agence Nationale de la Recherche (ANR-11-BSV3-018), La Ligue Contre le Cancer (Labellisée 2006-2012), and Institut National du Cancer (INCa #2011-144) and funding from Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, and Université de Strasbourg. B. Heizmann received postdoctoral funding from Fondation ARC.

The authors have no conflicting financial interests.

Submitted: 19 August 2013
Accepted: 8 November 2013

REFERENCES

Alkhatib, A., M. Werner, E. Hug, S. Herzog, C. Eschbach, H. Faraidun, F. Köhler, T. Wossning, and H. Jumaa. 2012. FoxO1 induces Ikaros splicing to promote immunoglobulin gene recombination. *J. Exp. Med.* 209:395–406. http://dx.doi.org/10.1084/jem.20110216

Amin, R.H., and M.S. Schlissel. 2008. Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nat. Immunol.* 9:613–622. http://dx.doi.org/10.1038/ni.1612

Baba, M., J.R. Keller, H.W. Sun, W. Resch, S. Kuchen, H.C. Suh, H. Hasumi, Y. Hasumi, K.R. Kieffer-Kwon, C.G. Gonzalez, et al. 2012. The folliculin-FNIP1 pathway deleted in human Birt-Hogg-Dubé syndrome is required for murine B-cell development. *Blood*. 120:1254–1261. http://dx.doi.org/10.1182/blood-2012-02-410407

Corfe, S.A., and C.J. Paige. 2012. The many roles of IL-7 in B cell development; mediator of survival, proliferation and differentiation. *Semin. Immunol.* 24:198–208. http://dx.doi.org/10.1016/j.smim.2012.02.001

Dupuis, A., M.P. Gaub, M. Legrain, B. Drenou, L. Mauvieux, P. Lutz, R. Herbrecht, S. Chan, and P. Kastner. 2013. Biclonal and biallelic deletions occur in 20% of B-ALL cases with IKZF1 mutations. *Leukemia*. 27:503–507. http://dx.doi.org/10.1038/leu.2012.204

Eilers, M., and R.N. Eisenman. 2008. Myc's broad reach. *Genes Dev.* 22:2755–2766. http://dx.doi.org/10.1101/gad.1712408

Engelmann, A., D. Speidel, G.W. Bornkamm, W. Deppert, and C. Stocking. 2008. Gadd45 beta is a pro-survival factor associated with stress-resistant tumors. *Oncogene*. 27:1429–1438. http://dx.doi.org/10.1038/sj.onc.1210772

Ferreirós-Vidal, I., T. Carroll, B. Taylor, A. Terry, Z. Liang, L. Bruno, G. Dharmalingam, S. Khadayate, B.S. Cobb, S.T. Smale, et al. 2013. Genome-wide identification of Ikaros targets elucidates its contribution to mouse B-cell lineage specification and pre-B-cell differentiation. *Blood*. 121:1769–1782. http://dx.doi.org/10.1182/blood-2012-08-450114

Fuxa, M., J. Skok, A. Souabni, G. Salvagiotto, E. Roldan, and M. Busslinger. 2004. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev.* 18:411–422. http://dx.doi.org/10.1101/gad.291504

Gachet, S., E. Genescà, D. Passaro, M. Irigoyen, H. Alcalde, C. Clémenson, S. Poglio, F. Pflumio, A. Janin, C. Lasgi, et al. 2013. Leukemia-initiating cell activity requires calcineurin in T-cell acute lymphoblastic leukemia. *Leukemia*. http://dx.doi.org/10.1038/leu.2013.156

Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 334:676–682. http://dx.doi.org/10.1038/334676a0

Harvey, R.C., C.G. Mullighan, I.M. Chen, W. Wharton, F.M. Mikhail, A.J. Carroll, H. Kang, W. Liu, K.K. Dobbin, M.A. Smith, et al. 2010. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. *Blood*. 115:5312–5321. http://dx.doi.org/10.1182/blood-2009-09-245944

Herzog, S., E. Hug, S. Meixlsperger, J.H. Paik, R.A. DePinho, M. Reth, and H. Jumaa. 2008. SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway. *Nat. Immunol.* 9:623–631. http://dx.doi.org/10.1038/ni.1616

Herzog, S., M. Reth, and H. Jumaa. 2009. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat. Rev. Immunol.* 9:195–205. http://dx.doi.org/10.1038/nri2491

Hobeika, E., S. Thiemann, B. Storch, H. Jumaa, P.J. Nielsen, R. Pelanda, and M. Reth. 2006. Testing gene function early in the B cell lineage in mb1-cre mice. *Proc. Natl. Acad. Sci. USA*. 103:13789–13794. http://dx.doi.org/10.1073/pnas.0605944103

Kastner, P., A. Dupuis, M.P. Gaub, R. Herbrecht, P. Lutz, and S. Chan. 2013. Function of Ikaros as a tumor suppressor in B cell acute lymphoblastic leukemia. *Am. J. Blood Res.* 3:1–13.

Kirstetter, P., M. Thomas, A. Dierich, P. Kastner, and S. Chan. 2002. Ikaros is critical for B cell differentiation and function. *Eur. J. Immunol.* 32:720–730. http://dx.doi.org/10.1002/1521-4141(200203)32:3<720::AID-IMMU720>3.0.CO;2-P

Lu, R., K.L. Medina, D.W. Lancki, and H. Singh. 2003. IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. *Genes Dev.* 17:1703–1708. http://dx.doi.org/10.1101/gad.1104803

Ma, S., S. Pathak, L. Trinh, and R. Lu. 2008. Interferon regulatory factors 4 and 8 induce the expression of Ikaros and Aiolos to down-regulate pre-B-cell receptor and promote cell-cycle withdrawal in pre-B-cell development. *Blood*. 111:1396–1403. http://dx.doi.org/10.1182/blood-2007-08-110106

Ma, S., S. Pathak, M. Mandal, L. Trinh, M.R. Clark, and R. Lu. 2010. Ikaros and Aiolos inhibit pre-B-cell proliferation by directly suppressing c-Myc expression. *Mol. Cell. Biol.* 30:4149–4158. http://dx.doi.org/10.1128/MCB.00224-10

Malin, S., S. McManus, C. Cobaleda, M. Novatchkova, A. Delogu, P. Bouillet, A. Strasser, and M. Busslinger. 2010. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat. Immunol.* 11:171–179. http://dx.doi.org/10.1038/ni.1827

Mandal, M., S.E. Powers, M. Maienschein-Cline, E.T. Bartom, K.M. Hamel, B.L. Kee, A.R. Dinner, and M.R. Clark. 2011. Epigenetic repression of the Igk locus by STAT5-mediated recruitment of the histone methyltransferase Ezh2. *Nat. Immunol.* 12:1212–1220. http://dx.doi.org/10.1038/ni.2136

Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. 267:1506–1510. http://dx.doi.org/10.1126/science.7878471

Mullighan, C.G., C.B. Miller, I. Radtke, L.A. Phillips, J. Dalton, J. Ma, D. White, T.P. Hughes, M.M. Le Beau, C.H. Pui, et al. 2008. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature*. 453:110–114. http://dx.doi.org/10.1038/nature06866

Park, H., K. Staehling, M. Tsang, M.W. Appleby, M.E. Brunkow, D. Margineantu, D.M. Hockenbery, T. Habib, H.D. Liggitt, G. Carlson, and B.M. Iritani. 2012. Disruption of Fnip1 reveals a metabolic checkpoint controlling B lymphocyte development. *Immunity*. 36:769–781. http://dx.doi.org/10.1016/j.immuni.2012.02.019

Pieper, K., B. Grimbacher, and H. Eibel. 2013. B-cell biology and development. *J. Allergy Clin. Immunol*. 131:959–971. http://dx.doi.org/10.1016/j.jaci.2013.01.046

Reynaud, D., I.A. Demarco, K.L. Reddy, H. Schjerven, E. Bertolino, Z. Chen, S.T. Smale, S. Winandy, and H. Singh. 2008. Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. *Nat. Immunol*. 9:927–936. http://dx.doi.org/10.1038/ni.1626

Sabbattini, P., M. Lundgren, A. Georgiou, C. Chow, G. Warnes, and N. Dillon. 2001. Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. *EMBO J*. 20:2812–2822. http://dx.doi.org/10.1093/emboj/20.11.2812

Schjerven, H., J. McLaughlin, T.L. Arenzana, S. Frietze, D. Cheng, S.E. Wadsworth, G.W. Lawson, S.J. Bensinger, P.J. Farnham, O.N. Witte, and S.T. Smale. 2013. Selective regulation of lymphopoiesis and leukemogenesis by individual zinc fingers of Ikaros. *Nat. Immunol*. 14:1073–1083. http://dx.doi.org/10.1038/ni.2707

Thompson, E.C., B.S. Cobb, P. Sabbattini, S. Meixlsperger, V. Parelho, D. Liberg, B. Taylor, N. Dillon, K. Georgopoulos, H. Jumaa, et al. 2007. Ikaros DNA-binding proteins as integral components of B cell developmental-stage-specific regulatory circuits. *Immunity*. 26:335–344. http://dx.doi.org/10.1016/j.immuni.2007.02.010

Trageser, D., I. Iacobucci, R. Nahar, C. Duy, G. von Levetzow, L. Klemm, E. Park, W. Schuh, T. Gruber, S. Herzog, et al. 2009. Pre–B cell receptor–mediated cell cycle arrest in Philadelphia chromosome–positive acute lymphoblastic leukemia requires *IKAROS* function. *J. Exp. Med*. 206:1739–1753. http://dx.doi.org/10.1084/jem.20090004

Uckun, F.M., H. Ma, J. Zhang, Z. Ozer, S. Dovat, C. Mao, R. Ishkhanian, P. Goodman, and S. Qazi. 2012. Serine phosphorylation by SYK is critical for nuclear localization and transcription factor function of Ikaros. *Proc. Natl. Acad. Sci. USA*. 109:18072–18077. http://dx.doi.org/10.1073/pnas.1209828109

Wang, J.H., A. Nichogiannopoulou, L. Wu, L. Sun, A.H. Sharpe, M. Bigby, and K. Georgopoulos. 1996. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity*. 5:537–549. http://dx.doi.org/10.1016/S1074-7613(00)80269-1

Winkler, T.H., F. Melchers, and A.G. Rolink. 1995. Interleukin-3 and interleukin-7 are alternative growth factors for the same B-cell precursors in the mouse. *Blood*. 85:2045–2051.