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Transcriptional regulation of the *Ikzf1* locus

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Key Points

- Multiple enhancers identified at the *Ikzf1* locus with shared and distinct epigenetic and transcriptional properties.
- Transcription factor networks that distinguish between LMPP-specific and T cell–specific *Ikzf1* enhancers.

Ikaros is a critical regulator of lymphocyte development and homeostasis; thus, understanding its transcriptional regulation is important from both developmental and clinical perspectives. Using a mouse transgenic reporter approach, we functionally characterized a network of highly conserved *cis*-acting elements at the *Ikzf1* locus. We attribute B-cell and myeloid but not T-cell specificity to the main *Ikzf1* promoter. Although this promoter was unable to counter local chromatin silencing effects, each of the 6 highly conserved *Ikzf1* intronic enhancers alleviated silencing. Working together, the *Ikzf1* enhancers provided locus control region activity, allowing reporter expression in a position and copy-independent manner. Only 1 of the *Ikzf1* enhancers was responsible for the progressive upregulation of Ikaros expression from hematopoietic stem cells to lymphoid-primed multipotent progenitors to T-cell precursors, which are stages of differentiation dependent on Ikaros for normal outcome. Thus, *Ikzf1* is regulated by both epigenetic and transcriptional factors that target its enhancers in both redundant and specific fashions to provide an expression profile supportive of normal lymphoid lineage progression and homeostasis. Mutations in the *Ikzf1* regulatory elements and their interacting factors are likely to have adverse effects on lymphopoiesis and contribute to leukemogenesis. (*Blood*. 2013; 122(18):3149-3159)

Introduction

Lymphocyte differentiation is dependent on nuclear factors acting as key regulatory nodes that control gene expression in a cell type– and stage-specific manner. A critical node in the lymphoid lineage regulatory circuit is the Ikaros family of zinc-finger DNA binding proteins, inactivation of which causes lymphocyte disorders and lymphoid malignancies.[1,2]

The first major role played by Ikaros proteins is manifested in the lymphoid-primed multipotent progenitor (LMPP).[3-5] Ikaros' affiliation with a higher-order epigenetic complex that contains disparate chromatin remodeling activities on one hand primes an early lymphoid lineage transcriptional signature, whereas on the other, it represses a stem cell–specific transcriptional signature.[6] Ikaros-deficient LMPPs are unable to undergo lymphoid differentiation. Instead they differentiate into the myeloid lineage while retaining significant stem cell–specific gene expression.[4,6] Importantly, restriction of a hematopoietic stem cell (HSC) into an LMPP is underscored by an increase in Ikaros expression.

The second major role played by Ikaros is at stages of T- and B-cell differentiation that express high levels of Ikaros mRNA and protein.[1,7] Reduction in Ikaros levels in double positive thymocytes and in pre-B cells is associated with aberrant differentiation and leukemic transformation in both mice and humans.[8-15]

Here, we evaluate the transcriptional mechanisms that control *Ikzf1* expression in the hematopoietic system. Using a mouse transgenic reporter approach, we establish the activities of 10 lymphoid-specific clusters of DNase I hypersensitivity sites (DHSs) previously mapped at the *Ikzf1* locus.[16] Our studies reveal that transcription of the *Ikzf1* locus is regulated by a network of epigenetic and transcription factors working together in a unique and redundant fashion to provide the appropriate levels of Ikaros expression needed for lymphocyte development.

Material and methods

Mice

Ikaros-green fluorescent protein (GFP) reporter lines (C57BL/6 × C3H) and *Ikzf1* bacterial artificial chromosome (Ik-BAC) transgenic lines (C57BL/6 × C3H) were generated in the Massachusetts General Hospital transgenic core facility. Mice were bred and maintained under specific pathogen-free conditions in the animal facility in Massachusetts General Hospital, Building 149. The mice were 4 to 12 weeks old at the time of analyses.

Generation of Ikaros-GFP reporter transgenic mice

All GFP transgenic reporters used in this study are based on the B-*p*-GFP.[16] Genomic regions J, D, E, F, G, H, and I (described in Table 1) were PCR

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Table 1. Regions of mouse-human homology identified on the *Ikzf1*

Conserved region	Total no. bp of homology (no. of subregions)	Percentage mouse-human homology in subregions (total)	Mouse mm9 (Chr11)	Human hg19 (Chr7)	Length (bp) of fragment used for a transgenic cassette	Tissue-specific DHS cluster in vicinity?
J	645 (1)	96	11575935-11576581	50333380-50334026	647	No
p*	632 (2)	97	11599576-11600207	50358080-50358643	442	DHS-C4
D†	424 (7)	93	11616550-11618284	50287655-50290663 (mm7 > hg18)	1735	DHS-C6
E‡	258 (2)	91	11625474-11626435	50418201-50419257	962	DHS-C7
F	204 (1)	86	11630128-11630533	50424272-50424659	406	DHS-C8
G	161 (1)	88	11633285-11633536	50427799-50427958	322	No
H§	204 (3)	95	11640927-11644360	50435678-50439913	3434	DHS-C9
I	529 (1)	96	11653307-11653987	50449525-50450219	681	No

Conserved regions listed in 5′ to 3′ order (top to bottom) according to their relative position on the *Ikzf1* locus.

*p was initially believed to be a promoter, based on the extent of cross-species conservation and the presence of TATA and CAAT box elements within the p sequence. However, transgenic cassettes using this fragment in the absence of untranslated exons 1a or 1b have failed to generate mice with lympho-myeloid specific reporter activity.

†Region D consists of seven subregions (length, % homology): D1 (45 bp, 89%), D2 (52 bp, 96%), D3 (78 bp, 94%), D4 (38 bp, 94%), D5 (67 bp, 97%), D6 (102 bp, 86%), and D7 (35 bp, 94%) (supplemental Table 3).

‡Region E consists of two subregions (length, % homology): E1 (134 bp, 94%) and E2 (122 bp, 88%).

§Region H consists of three subregions (length, % homology): H1 (115 bp, 93%), H2 (47 bp, 95%), and H3 (40 bp, 92%).

amplified from an *Ikzf1* locus containing BAC vector (Ik-BAC-3; Genome Systems, Inc., St. Louis, MO) using specific primers described in supplemental Table 5 on the *Blood* Web site. More detailed strategies for vector constructions and generation of mice are described in supplemental Methods.

Generation of BAC transgenic mice

Detailed strategies for construction of Ik-BAC-hCD2, Ik-BAC-lDl, and Ik-BAC-ΔD vectors, generation of mice, screening for transgene positive founders, and copy number determination are described in supplemental Methods (see also "Constructs for recombination templates for BAC engineering").

Flow cytometric analysis

Antibodies, their specific clones, and flow cytometers (BD) used are described in supplemental Methods. Cell sorting was performed on a Moflo (Dako Cytomation). Data analysis was performed using FlowJo software (Tree Star, Inc.).

GFP reporter expression analysis in the brain

Brains were isolated from P0 embryos and photographed in whole mount in the green fluorescence channel. The samples were cleaved at the posterior edge of the basal ganglia and fixed overnight in 4% paraformaldehyde in PBS at 4°C and then dehydrated through graded sucrose, embedded in OCT, and cryosectioned.

Chromatin immunoprecipitation sequencing analysis

Chromatin immunoprecipitation sequencing (ChIP-seq) for RNA polymerase II (RNApII) CTD phospho Ser5, HEB, H3K4me1, me2, me3, me36, and H3K9Ac in wild-type thymocytes was previously described.[10,17] Briefly, 10^7 to 10^8 thymocytes were formaldehyde-fixed and sonicated to an average size of 300 bp. ChIP-seq for T cell factor 1 (TCF-1) (a kind gift of Dr Kawamoto), RNApII CTD phospho Ser2 (ab5095), and the unphosphorylated form of RNApII (8WG16) was performed as previously described.[10,17,18] ChIP-seq data for runt-related transcription factor 1 (Runx1)[19] were obtained from the Gene Expression Omnibus database under accession number SRR364255. ChIP-seq for E26 avian leukemia oncogene 1, 5′ domain (Ets-1), myelocytomatosis oncogene (c-Myc), GATA binding protein 1 (GATA-1), GATA binding protein 2 (GATA-2), and T cell acute lymphocytic leukemia 1 (Tal-1) were obtained from the Encyclopedia of DNA Elements (ENCODE) project (supplemental Table 7).[20] Sequence reads were aligned to the mouse genome assembly mm9 with bowtie 0.12.7,[21] allowing up to 2 mismatches and keeping only uniquely aligned reads. Tag density tracks and peak calling were generated with MACS 1.4.2.[22]

Results

Enhancer-associated epigenetic marks at the *Ikzf1* lymphoid-specific DHS

Mapping of lymphoid-specific clusters of DHSs at the *Ikzf1* locus has previously identified 10 putative regulatory regions[16] (Figure 1). The majority of these sites were also recently identified by genomewide DHS mapping in thymocytes, splenocytes, and B cells generated by the ENCODE project[20] (supplemental Figure 1; supplemental Tables 6 and 8). In proximity to these DHS sites were regions of extensive conservation between the mouse and human *Ikzf1* (*IKZF1*). Seven intronic (p, D, E, F, G, H, and I) and 1 upstream (J) conserved regions were identified, and 5 (D-H) were located within the ~40-kb intron between exons 3 and 4 (>86%; Table 1; Figure 1).

To gain insight into the types of regulatory elements associated with the *Ikzf1* conserved DHSs, the immediate chromatin environment was examined by ChIP of histone modifications coupled with high-throughput sequencing (ChIP-Seq) in thymocytes.[17] We first examined the methylation status of H3K4 that correlates with both transcriptionally poised and transcriptionally active promoters and enhancers.[23] H3K4me1 exhibited a wide distribution over the entire *Ikzf1* locus. In contrast, H3K4me2 and H3K4me3 were specifically enriched at the *Ikzf1* putative regulatory sites and not throughout the locus (J, p, D, E, F, G, H, and I). Nonetheless, their distributions differed, with H3K4me3 being highly enriched at promoters and relatively depleted at the 5′ and intronic DHS, whereas H3K4me2 was evenly distributed at all these sites. Similar to H3K4me3, H3K9Ac displayed strong promoter enrichment and lower enrichment at other regulatory sites. Similar to H3K4me2, the enhancer marks p300 and H3K27Ac[20] showed a nonpromoter distribution.

Transcriptionally active enhancers at the *Ikzf1* locus were further evaluated by examining the distribution of the preinitiation and elongation forms of RNApII.[24] The S5 and S2 phosphorylated forms of RNA polymerase, as well as the transcriptional elongation mark H3K36me3, were distributed throughout the *Ikzf1* locus (Figure 1). In contrast, the unphosphorylated form of RNA polymerase (recognized by the 8WG16 antibody) that marks the preinitiation phase of transcription and active enhancers was only detected at the *Ikzf1* regulatory clusters.

Thus, the differential distribution of histone codes, chromatin modifiers, and transcription factors at several of the *Ikzf1* conserved

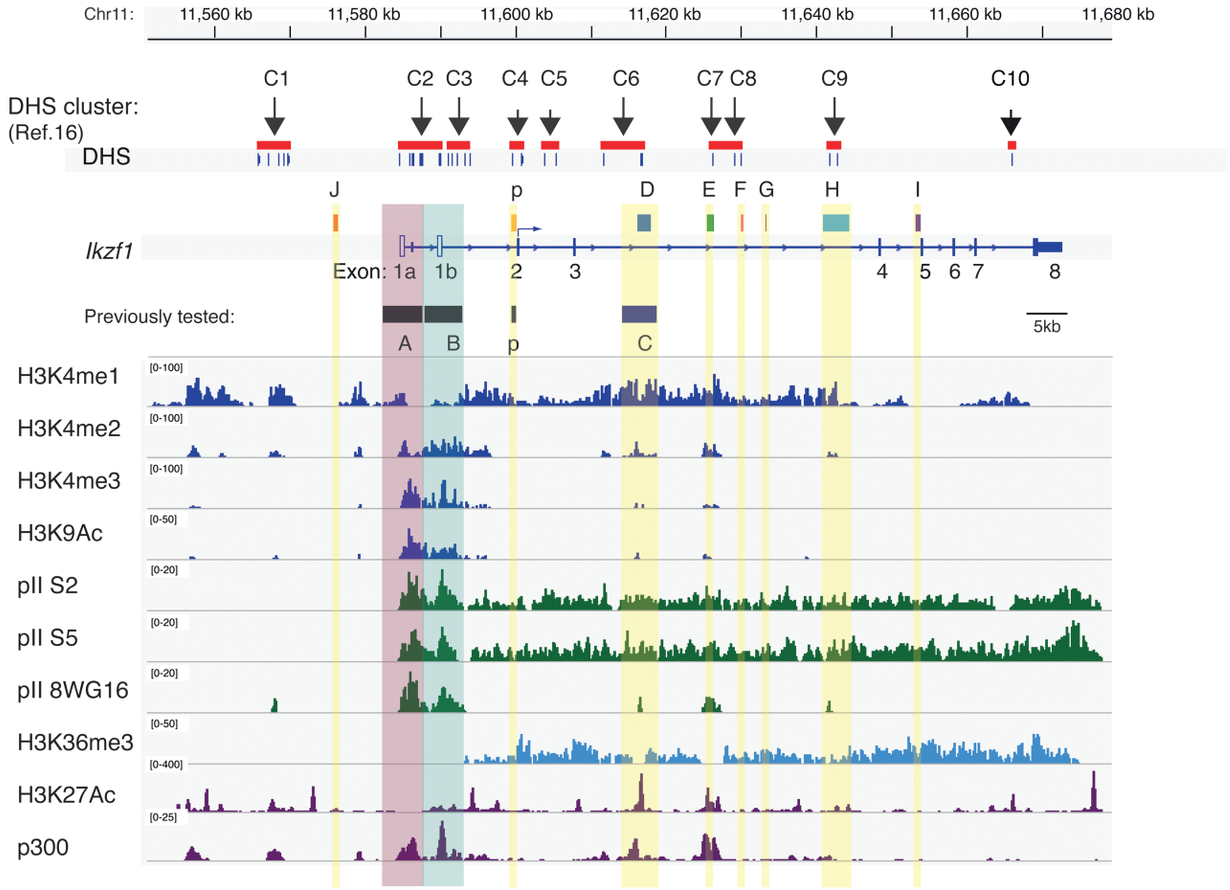

Figure 1. Epigenetic demarcation of *Ikzf1* regulatory regions. The location of 10 previously described lymphoid-specific DHS clusters[16] and noncoding regions with extensive mouse-human sequence conservation are shown at the *Ikzf1* locus. Individual DHS and DHS clusters are indicated by short vertical bars or red bars. On the *Ikzf1* locus, the untranslated exons 1a and 1b associated with distinct promoter activities are denoted as unfilled rectangles and the 7 translated exons 2 to 8 as solid rectangles. Enrichment peaks for histone H3 modifications (H3K4me1, me2, me3, H3K9/14Ac, and H3K36me3) and for the RNA Pol II (pII) initiation (8WG16) and elongation (S5, S2) forms at the *Ikzf1* locus are displayed by the Integrative Genomics Viewer (IGV) 2.1 using the mouse genome database mm9. Enrichment peaks for H3K27Ac and p300 were deduced from ENCODE data (supplemental Table 8).[20]

DHS clusters supports their potential role as transcriptional enhancers in regulating Ikaros expression during development.

Epigenetic and transcriptional properties of the *Ikzf1* enhancers

The putative *Ikzf1* enhancers were tested for their ability to promote transcription of a GFP reporter from the main *Ikzf1* (B-p) promoter (Figures 1 and 2A).[16] Reporter cassettes were generated in which the putative enhancer orientation relative to the promoter was preserved, as in the *Ikzf1* locus. For each reporter cassette, multiple mouse transgenic lines were generated and tested for GFP expression in peripheral blood leukocytes (PBLs). The number of lines for each reporter and the transgene copy number are provided (supplemental Figures 2 and 3; supplemental Table 1).

The activity of the *Ikzf1* regulatory regions in counteracting local chromatin silencing and in promoting transcription were evaluated by estimating the number of GFP expressing transgenic lines (>1% expression in PBLs), the expression in PBL subsets, and the average level of reporter expression. The majority of transgenic lines generated by these enhancer-driven reporters exhibited GFP expression in PBL (Figure 2A-B). This was a significant increase ($P < .05$) over the small fraction of GFP-expressing founders obtained with the just-promoter-driven parental reporter (Figure 2A; B-*p*-GFP, 11%).[16] Among the *Ikzf1* regulatory regions, J, D, and E displayed the strongest enhancer activity, supporting expression in 88% to 93% of the transgenic lines, whereas F, I, and H were weaker, supporting expression in 53% to 75% of the transgenic lines (Figure 2A; supplemental Figures 2 and 3; supplemental Table 1).

Reporter expression in PBLs was highly variegated (Figure 2B), indicating that although the *Ikzf1* enhancers could initially counter-restrictive chromatin at the site of reporter integration, they were unable to maintain transcriptionally permissive chromatin within the *Ikzf1*-expressing PBL subsets. For example, although the B-*p*-GFP-D reporter was expressed in 93% of its transgenic lines ($P < 5 \times 10^7$), on average, expression was detected in only 42% of PBLs. On the other hand, although the B-*p*-GFP-H reporter was expressed in a smaller fraction (53%) of founder lines, expression was seen in a larger fraction (48%) of PBLs, indicating a stronger potential in maintaining transcriptionally permissive chromatin (Figure 2B).

The *Ikzf1* enhancers were further evaluated by measuring the level of reporter expression. Notably, the mean fluorescence intensity (MFI) of GFP-expressing cells from the D enhancer-reporter lines surpassed those of all other reporters, whether measured from all transgenic lines or from expressing transgenic lines (Figure 2C, average shown as gray or black line, respectively). The considerable difference

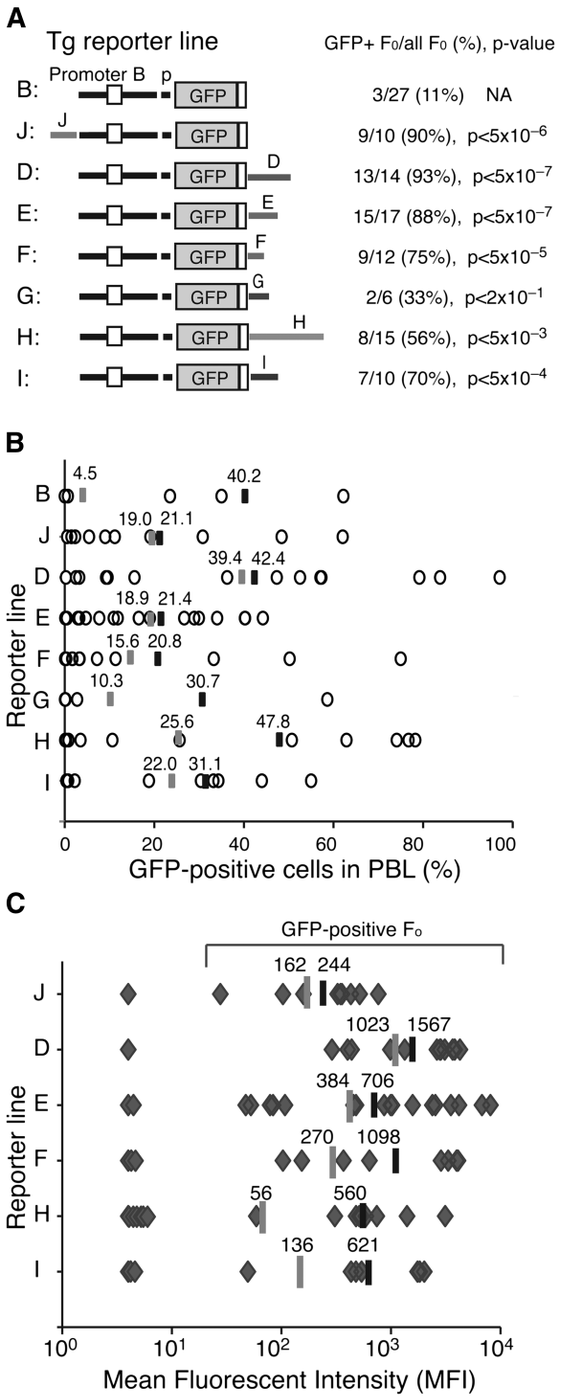

Figure 2. Enhancer activities associated with the *Ikzf1* regulatory sites. (A) Diagrams of the reporter constructs used in this study are shown on the left. The number of GFP-expressing founders is displayed over the total number of founders obtained for each reporter. The statistical significance of the difference (increase) in GFP-expressing founder lines compared with the parental B-*p*-GFP lines was provided by χ^2 analysis and is shown as a *P* value. (B) The percentage of GFP$^+$ PBLs assessed by flow cytometry is depicted for each founder line as circles. The average percentage of GFP$^+$ PBLs for each transgenic reporter was calculated for either all GFP-expressing founders (black bar) or all founders (gray bar). (C) The MFI of GFP$^+$ PBLs was estimated for each founder line (gray diamonds) of every transgenic reporter as described in panel B.

in expression provided by the average MFI of all transgenic vs expressing transgenic lines for F, H, and I suggested that these enhancers were potent in stimulating transcription but only in permissive chromatin. In contrast, although the upstream J enhancer was weak in stimulating transcription, it was very efficient in counteracting repressive chromatin, providing a greater number of low-expressing transgenic lines.

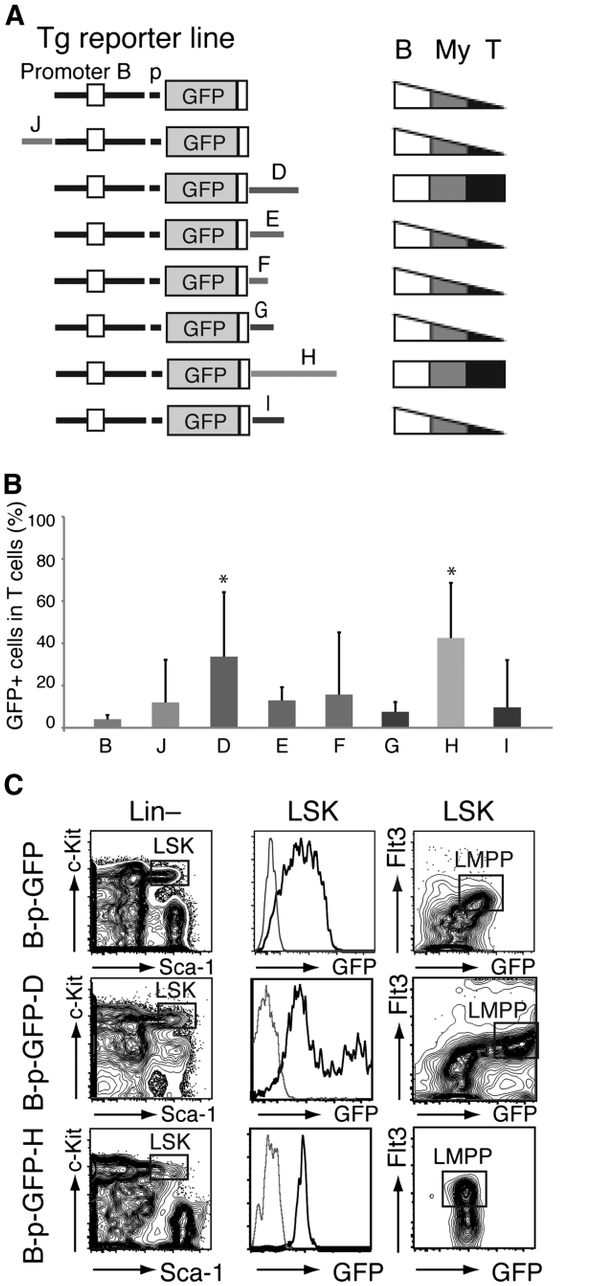

Figure 3. Cell type specificity of the *Ikzf1* enhancers. (A) Diagrams of the reporter constructs used in this study are shown on the left. Patterns of lineage-specific GFP expression in PB$^-$ B cells (B220$^+$, B), T cells (TCRβ$^+$, T), and myeloid (Mac-1$^+$, My) cells are summarized on the right. (B) *Ikzf1* enhancer activity in T cells. Error bars (standard deviation) indicate variegation of GFP expression among the different founder lines generated by each enhancer-based reporter. The significance in the difference of GFP expression among GFP-reporter lines was assessed by Student *t* test; $*P < 5 \times 10^{-3}$. (C) Enhancer D and H activity in the HSC/MPP-enriched LSK (Lin$^-$c-Kit$^+$Sca-1$^+$), as determined by expression of B-*p*-GFP-D and -H reporters compared with the parental B-*p*-GFP reporter. The LMPP is defined as LSK Flt3$^+$ (square gate). GFP expression in the LSK is shown as a histogram or together with Flt3 expression as a contour plot. Thin gray line histogram represents a reporter negative control.

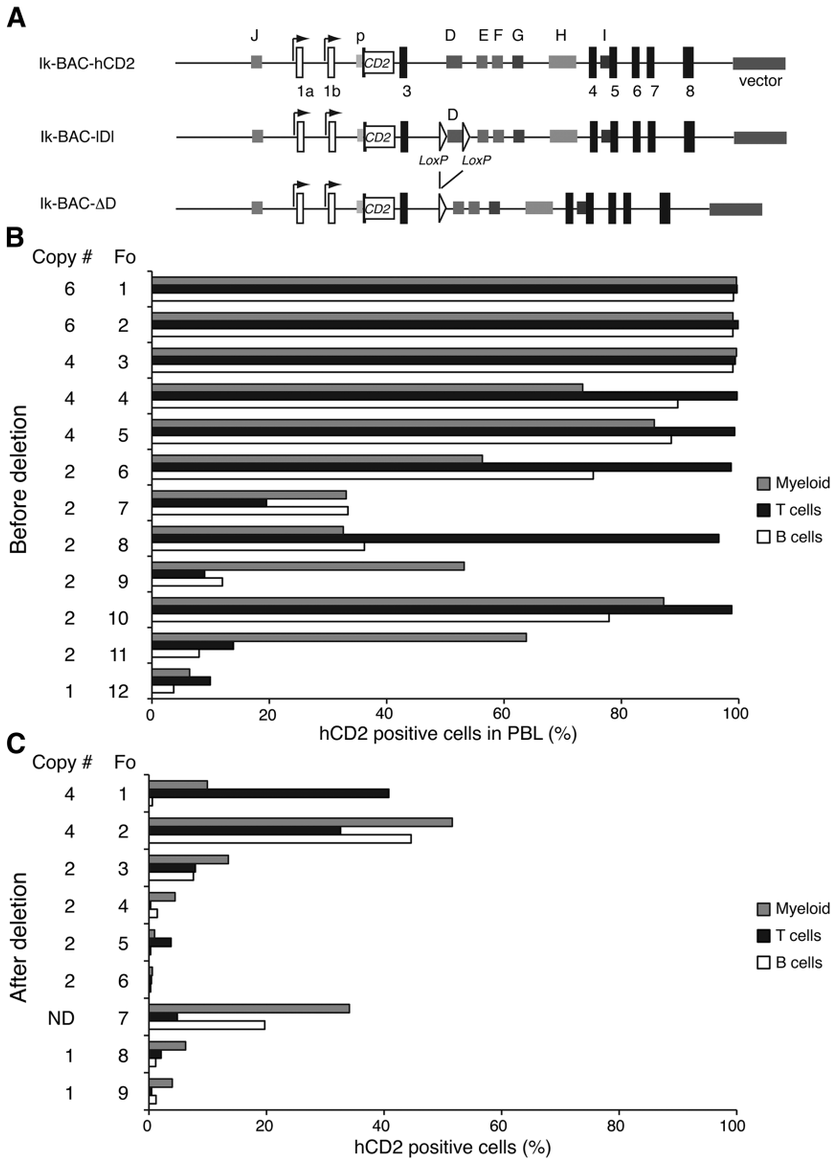

Figure 4. Enhancer D is required for normal transcription at the *Ikzf1 locus* (A) Diagram of the *Ikzf1* BAC transgenic reporter constructs. The first translated exon (exon 2) in the *Ikzf1* BAC clone was replaced by the *human CD2* (hCD2) reporter gene (white rectangle) inserted at the initiation codon (Ik-BAC-CD2). Two flanking *loxP* sites (white triangles) were inserted 5′ and 3′ of the D region (Ik-BAC-IDI). The D region was deleted from Ik-BAC-IDI by *Cre* recombinase to generate Ik-BAC-ΔD construct. (B) Reporter expression in the PBL from Ik-BAC transgenic lines with intact enhancer D region. (C) Reporter expression in PBL from Ik-BAC-ΔD transgenic lines. The copy number is noted besides each founder generated from either the Ik-BAC-CD2 or the Ik-BAC-DL line. The percentage of $hCD2^+$ myeloid cells (gray bars), T cells (black bars), and B cells (white bars) was determined for each founder animal by flow cytometry with antibodies to Mac-1, B220, and TCRβ, respectively. N.D., not determined.

Thus, analysis of transgenic reporters revealed the presence of 2 types of *cis*-acting elements associated with *Ikzf1* enhancers (supplemental Table 1). One type countered restrictive chromatin and position effect variegation (PEV) as documented by an increase in both the number of expressing founders and reporter-expressing PBLs. These elements likely support recruitment of epigenetic factors that promote a transcriptionally permissive chromatin state. The second type was potent in stimulating transcription in permissive chromatin, in line with the activity of a classical enhancer that functions by promoting the initiation or elongation phase of the RNApII cycle.

Cell type specificity of the *Ikzf1* enhancers

The *Ikzf1* enhancers were next evaluated for their cell type–specific effects on reporter expression (Figure 3A-B; supplemental Figures 2, 3, and 5). The expression pattern of their transgenic lines was examined for deviations from the "high B/low T/intermediate myeloid" profile provided by the parental just-promoter-driven reporter on which these enhancers were tested (Figure 3A-B; B-*p*-GFP).[16] PBLs from each founder line were examined for GFP expression in B cells ($B220^+$), T cells ($TCR\beta^+$), and myeloid cells ($Mac\text{-}1^+$) in peripheral blood by flow cytometry (Figure 3A; supplemental Figures 2, 3, and 5).

The majority of founder lines generated by the J, E, F, G, and I reporter cassettes displayed a similar expression pattern to the parental cassette (Figure 3A-B; supplemental Figures 2 and 5; supplemental Table 1). In sharp contrast, a significantly large number of the D and H founder lines expressed GFP in a major fraction of T cells compared with the parental reporter (34% and 43% compared with 4%; Figure 3A-B; supplemental Figures 2 and 3; supplemental Table 1). The average proportion of GFP^+ myeloid cells was also highest in the D transgenic lines (37% vs 22%; supplemental Figures 2 and 3; supplemental Table 1). Of all the *Ikzf1* enhancers tested here, D was the only one capable of stimulating reporter expression in the LMPP, the first step in lymphoid lineage restriction, above the basal levels detected in the HSC-enriched population (Figure 3C, LSK $Flt3^+$ vs LSK $Flt3^-$).[4]

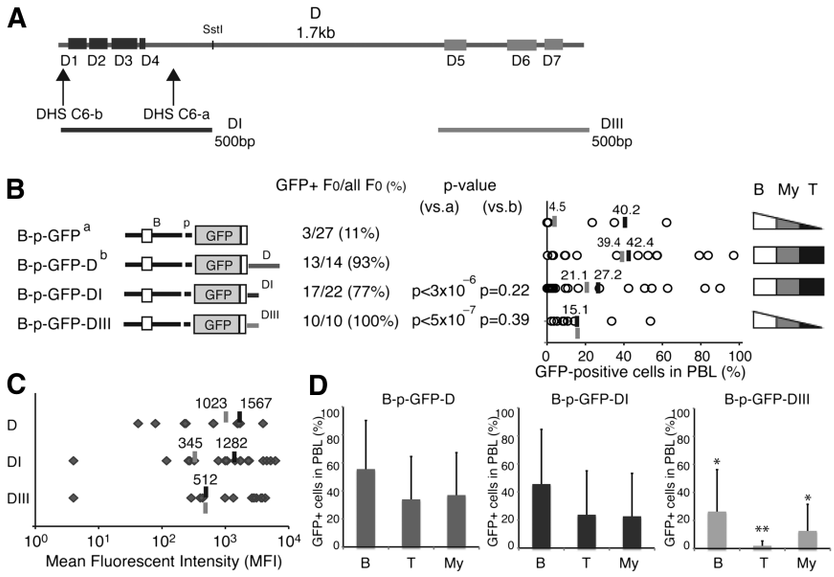

Figure 5. T cell–specific *cis*-acting elements in enhancer D. (A) Areas of sequence conservation (D1-D7) within the 1.7-kb enhancer D are shown as rectangles. Horizontal lines below the D region depict the two 500-bp DI and DIII subregions used in transgenic reporters. (B) Comparison of reporter expression between the B-*p*-GFP-DI, B-*p*-GFP-DIII, B-*p*-GFP, and B-*p*-GFP-D founder lines. The reporter constructs used to generate these founder lines are depicted on the left. The number of GFP-expressing founders is displayed over the total number of founders generated. The statistical significance of the difference (increase or decrease) in the proportion of GFP-expressing founder compared with the parental B-*p*-GFP line (a) or B-*p*-GFP-D (b) was provided by χ^2 analysis and is shown as a *P* value. The percentage of GFP-positive PBLs is depicted for each founder line (circle). The average percentage of GFP^+ PBLs for each transgenic reporter was calculated for either all GFP-expressing founders (black bar) or all founders (gray bar). The cell type specificity of each reporter is depicted on the right. (C) The MFI of GFP^+ PBLs from B-*p*-GFP-D, B-*p*-GFP-D-I, and B-*p*-GFP-D-III transgenic lines was calculated for each founder (gray diamonds). Gray lines indicate the average MFI among all founders, and black lines denote the average MFI among the GFP-expressing founders. (D) Lineage-specific GFP expression in the PBLs from B-*p*-GFP-D, B-*p*-GFP-DI, and B-*p*-GFP-DIII founders. The average percentage of GFP^+ cells within peripheral blood B cells (B), T cells (T), and myeloid cells (My) is shown. The significance in the difference of expression among GFP reporters was assessed by Student *t* test; $*P < 5 \times 10^{-2}$; $**P < 5 \times 10^{-3}$. Error bars (standard deviation) indicate variegation of GFP expression among the different founder lines made by each enhancer-based reporter.

Thus, whereas all 6 of the *Ikzf1* enhancers (J, D, E, F, H, and I) were active in B and myeloid cells, 2 (D and H) were also active in T cells and were likely responsible for high expression of *Ikzf1* in thymocytes required for normal maturation and homeostasis.[1,9,17,25,26] One of the 2 *Ikzf1* T cell–specific enhancers (D) was active in the LMPP and was associated with both an increase in Ikaros expression and induction of lymphoid lineage differentiation potential.[4,6]

Enhancer D plays a nonredundant role in the transcriptional regulation of *Ikzf1*

Given the potential importance of the enhancer D, we examined how its deletion affected transcriptional activity at the *Ikzf1* locus. An *Ikzf1* BAC clone with the human CD2 (hCD2) reporter inserted in exon 2 (Ik-BAC-hCD2) was engineered to contain *loxP* recognition sites flanking the 1.7-kb D region and was tested for expression in lymphoid and myeloid cells prior to (Ik-BAC-lDl) and after Cre-mediated deletion (Ik-BAC-ΔD) (Figure 4A). Because these BAC vectors were injected into fertilized eggs in a circular form, we presumed that transgenic lines with ⩾3 copies of the transgene had ⩾1 intact BAC clone. All of the founders obtained with Ik-BAC-hCD2 and Ik-BAC-lDl were expressed in ~90% of T, B, and myeloid cells, even when 2 copies of the BAC transgene were present (Figure 4B). In contrast, in all of the founders obtained with Ik-BAC-ΔD, a highly variegated expression of the hCD2 reporter was detected in all cell types, irrespective of the copy number of the transgene (Figure 4C). Thus, enhancer D is critical in counteracting repressive chromatin effects at the *Ikzf1* locus and maintaining transcription at high levels, an activity that cannot be substituted by the other *Ikzf1* enhancers.

Characterization of the *cis*-acting elements of the *Ikzf1* enhancer D

We further characterized the *cis*-acting elements responsible for enhancer D activities. The 1.7-kb region encompassing this enhancer contained 7 smaller areas (D1-D7) of strong mouse-human conservation (86%-96%) that ranged in size from 37 to 103 bp (Figure 5A; supplemental Figure 6; supplemental Table 3). The first 4 areas (D1-D4) were clustered at the 5′ end and were associated with the 2 DHS-C6 sites (Figure 5A; DHS-C6-a and DHS-C6-b), whereas the other 3 sites (D5-D7) covered a total of 394 bases located at the 3′ end (Figure 5A). Two 500-bp fragments encompassing the D1 to D4 (DI) and D5 to D7 (DIII) sequences, respectively, were cloned into the B-*p*-GFP cassette and evaluated for activity compared with the 2 parental vectors (Figure 5B; B-*p*-GFP and B-*p*-GFP-D).

Both enhancer subdomains generated a high number of expressing founder lines that were comparable to that of the intact enhancer D (Figure 5B). However, within the expressing populations, reporter variegation was greater compared with that of the intact enhancer. On average, 42% of PBLs from the D founder lines expressed GFP but only 27% or 15% of PBL from the DI or DIII founders expressed GFP, respectively (Figure 5B; supplemental Table 2).

The level of reporter expression supported by the 2 enhancer D subdomains differed greatly, those by DIII being much lower compared with DI or with the intact parental enhancer (Figure 5C-D; MFI: 512 vs 1282 or 512 vs 1567). Expression supported by DI was comparable to that of the intact enhancer (Figure 5C; MFI: 1282 vs 1567). Furthermore, DI but not DIII was able to confer expression in a substantial fraction of T cells (Figure 5B,D; supplemental Figure 4; supplemental Table 2). By these criteria, the DI subregion had similar

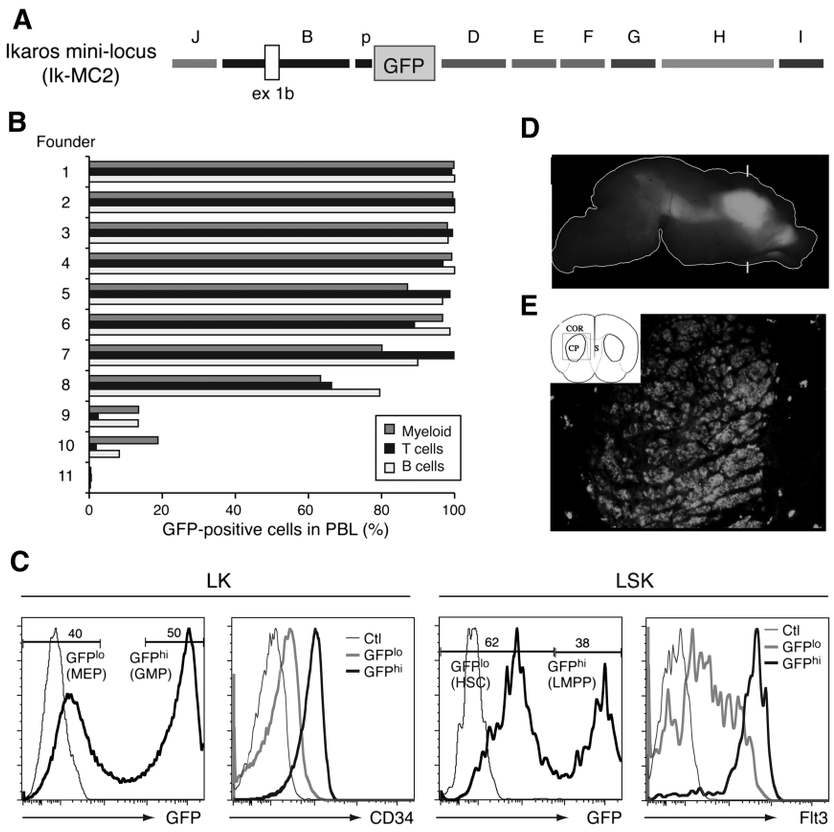

Figure 6. Combined activity of *Ikzf1* regulatory elements in lympho-myeloid and neuronal cells. (A) Diagram of the Ik-MC2 construct. (B) GFP reporter expression in PBL subsets of the Ik-MC2 founder lines. The percentage of GFP^+ myeloid cells (gray bars), T cells (black bars), and B cells (white bars) was determined for each founder line by fluorescence-activated cell sorter (FACS) analysis. (C) GFP expression in the HSC-enriched (LSK) and erythro-myeloid ($LK\text{-}Lin^-c\text{-}Kit^+Sca\text{-}1^-$) progenitor populations of the bone marrow. CD34 and Flt3 expression in LSK and LK GFP high (hi) and low (lo) subsets are shown. Thin line histogram represents FACS staining of a reporter or marker negative control. (D) Lateral view of GFP expression in the P0 brain in whole mount. The outline of the brain is shown as a white line. Bright GFP expression is observed in the basal ganglia. No fluorescence was observed in wild-type controls under these conditions. Brackets mark the approximate plane of the frontal section shown in panel E. (E) Schematic view of the section indicating the cortex (COR), caudate putamen (CP), and septum (S) and the area shown in the photograph (box). GFP expression is observed in the caudate putamen. With the exception of scattered cells elsewhere, GFP is not observed in surrounding tissue.

transcriptional and cell type–specific properties as the full-length enhancer D.

Combination of *Ikzf1* enhancers functions as a locus control region

When tested individually, the *Ikzf1* enhancers were capable of appreciable but not complete alleviation of PEV on reporter expression (Figure 2). This suggests that the combined enhancer activities, as encountered in the *Ikzf1* locus, are required for proper Ikaros expression in lympho-myeloid cells.

To test this hypothesis, we generated a transgenic reporter that combined most of these elements, preserving their orientation as in the endogenous locus (Ikaros mini-regulatory locus [Ik-MC2]; Figure 6A). Eight of 11 transgene positive founders exhibited GFP reporter expression in almost 100% of B cells, T cells, and myeloid cells in PBLs (Figure 6B). The 3 founders with low reporter expression may be due to integration of an incomplete transgenic reporter in the genome. Thus, the combined activity of regulatory elements present in the Ik-MC2 cassette can counteract PEV and provide an endogenous Ikaros-like pattern of expression in PBLs (Figure 6B).

We also evaluated the expression profile of the Ik-MC2 cassette in the HSCs and in early lineage restricted progenitors. This was very similar to that previously described for the enhancer D–containing reporters (Figure 3C; B-*p*-GFP-D and B-*p*-GFP-C).[4] The bimodal expression of the GFP reporter supported by the Ik-MC2 cassette separated the LMPP from the multipotent HSC (Figure 6C; LSK $Flt3^{++}$: GFP^{hi} from LSK $Flt3^{neg\text{–}lo}$: GFP^{lo}). It also segregated granulocyte-macrophage progenitors from megakaryocyte-erythrocyte progenitors in a mixed erythro-myeloid progenitor population (Figure 6C; LK $CD34^+$: GFP^{hi} from LK $CD34^{neg\text{-}lo}$: GFP^+).[4]

Ikzf1 is also expressed is in the developing striatum,[27,28] where it may play a role in neural progenitor function.[29,30] In contrast to enhancer D or other *Ikzf1* enhancers tested in isolation, their combined activity supported GFP expression in the striatum (Figure 6D-E). Sections through this region of the brain revealed expression throughout the caudate putamen similar to that exhibited by the endogenous *Ikzf1* locus (Figure 6D-E).

Thus, the combination of 9 of 10 of the *Ikzf1* conserved regions (J, B, p, D, E, F, G, H, and I) in a miniregulatory locus ensured gene expression in cells of the lympho-myeloid and neuronal lineage in a manner that resembled the cell type specificity of the endogenous *Ikzf1* locus and that was unaffected by local chromatin silencing effects.

Network of transcription factors regulating *Ikzf1* expression

To obtain insight into the transcription factors involved in *Ikzf1* regulation, motif search for transcription factor binding sites at enhancers D and H was performed. Enhancer D was specifically enriched with binding sites for transcription factors expressed in early hematopoietic progenitors such as Runx (*Runx1-3*), Homeobox A9 (*Hoxa9*), special AT-rich sequence binding protein 1 (Satb1), interferon regulatory factors (*Irf1, Irf4*), CCAAT/enhancer binding proteins (*Cebpa, Cepbb*), and myocyte enhancer factor 2C (*Mef2c*) (Figure 7B; supplemental Table 7D-H). These early lympho-myeloid transcription factor binding sites were not present in enhancer H. Nonetheless, several shared factor binding sites for transcription factors with previously reported activity in T cells were present in enhancers D and H, such as E2A (*Tcf3*), Ets-1 (*Ets1*), Tal-1 (*Tal1*), and Ikaros (*Ikzf1*) (supplemental Table 7D,H; Figure 7B). Further dissection of D into the DI and DIII subregions provided

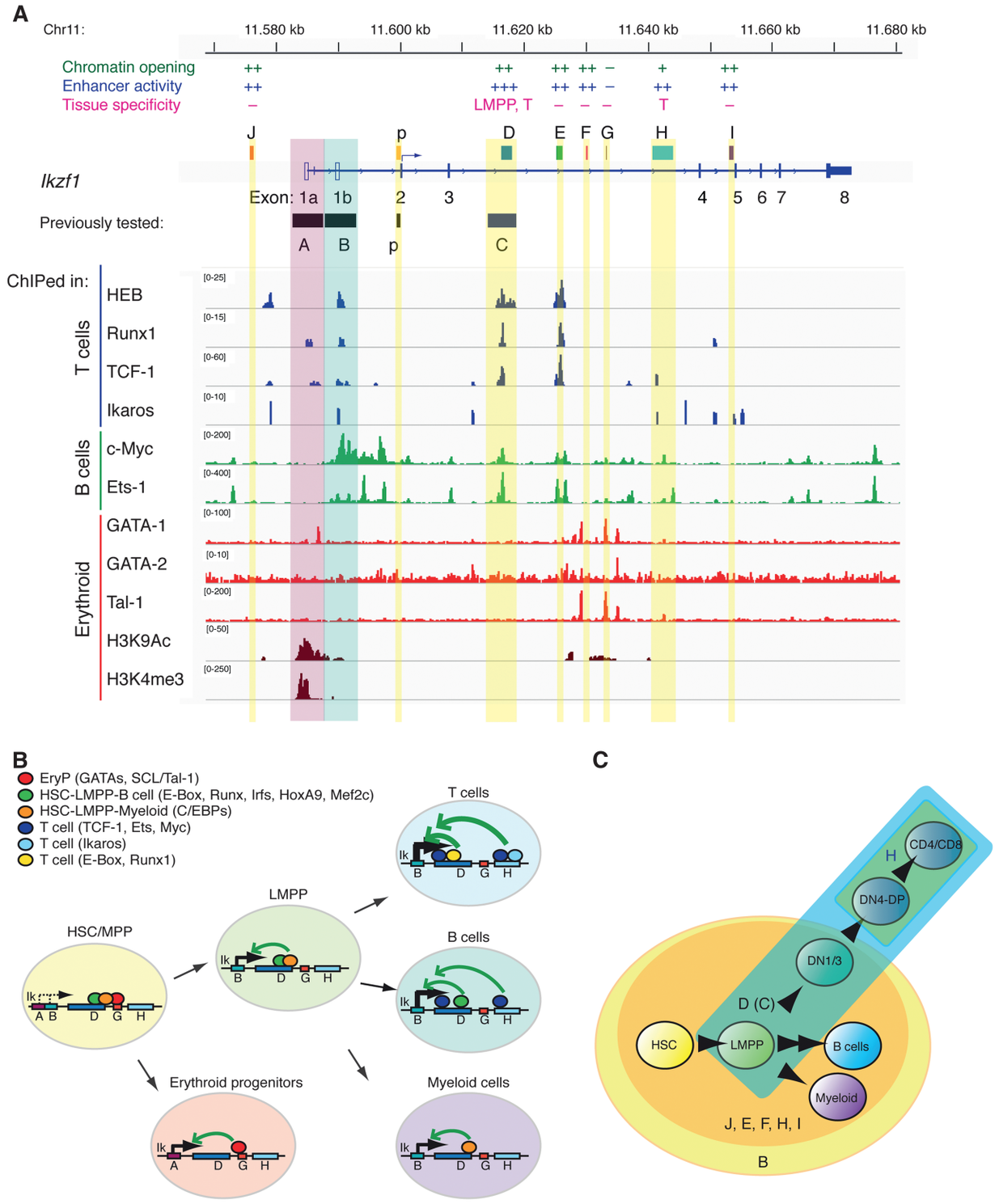

Figure 7. Network of transcription factors targeting *Ikzf1* enhancers. (A) TF enrichment peaks at the *Ikzf1* locus in thymocytes (T cells), CH12 (B cells), and erythoid precursors were visualized by the IGV browser. A summary of regulatory activities is provided above respective enhancer regions at the *Ikzf1* locus. (B-C) A model of *Ikzf1* regulation. (B) *Ikzf1* enhancer-promoter interactions during hematopoiesis. Erythroid-specific factors binding at enhancer G enable interactions with the myeloid-specific promoter A and Ikaros expression during early erythropoiesis. Lympho-myeloid–specific factors binding at enhancers D and H support interactions with the lympho-myeloid–specific promoter B and Ikaros expression during lymphoid and myeloid differentiation. Lineage-specific transcription factors at these sites are depicted as color-coded circles. Arrows indicate potential interactions between cell type–specific enhancers and promoters supporting *Ikzf1* expression at appropriate developmental stages. (C) *Ikzf1* regulatory region activity during lymphopoiesis. The *Ikzf1* lympho-myeloid promoter B, although active from the HSCs through B cell and myeloid differentiation, requires input from an enhancer to overcome the restrictive chromatin effects and PEV (yellow circle). Enhancers J, D(C), E, F, H, and I counteract PEV and raise *Ikzf1* gene expression (orange circle). For *Ikzf1* expression past the DN stage of T-cell development, input is required from enhancers D(C) (blue) and H (green). Induction of *Ikzf1* expression in the LMPP to the level required for lymphocyte differentiation is dependent on enhancer D(C) activity (blue).

independent evidence that the progenitor and T cell–specific activities of enhancer D resided within the DI subregion (supplemental Table 7DI-DIII; Figure 7B).

Transcription factors with putative binding sites in the *Ikzf1* regulatory elements were further evaluated by analysis of ChIP-seq data at the *Ikzf1* locus[17,19,31] (Figure 7A). Many of the lymphoid lineage pioneering factors such as Ikaros, HEB, Runx1, and TCF-1 bound at multiple sites in the *Ikzf1* locus. Enhancer D supported strong enrichment of a multitude of transcription factors expressed from the early to the later stages of lymphoid differentiation (E-box/HEB, Runx1, TCF-1, Ets-1, c-Myc), consistent with the enhancer's broad activity from the early progenitors to later stages in lymphocyte differentiation and its key role in the *Ikzf1* regulation. Interestingly, the neighboring enhancer E displayed a similar to D enrichment of transcription factors, although its in vivo activity was more restricted. The H enhancer with activity only in T cells had a limited repertoire of transcription factors that included Ikaros, TCF-1, Ets-1, and c-Myc. Finally, all the lymphoid promoting transcription factors bound to the lympho-myeloid promoter B, whereas binding of the erythroid specific transcription factor GATA-1 was detected in the vicinity of the myeloid-specific promoter A, which was specifically marked by H3K4me3 and H3K9Ac in erythroid progenitors. Analysis of erythroid transcription factor such as GATA-1, GATA-2, and SCL/Tal-1 (*Tal1*) identified enrichment at F and G regions in the vicinity of H3K9Ac, suggesting that these may be enhancers responsible for supporting Ikaros expression in erythroid progenitors. (Figure 7A-B).

Discussion

The transcriptional regulation of the *Ikzf1* gene is key to our understanding of the mechanisms that control normal lymphocyte differentiation and its aberrant manifestations. Here we show that *Ikzf1* expression in the hematopoietic system is controlled by a network of *cis*-acting elements that is spread over a 120-kb genomic region with many shared but also unique epigenetic and transcriptional regulatory features.

A comparative study of previously established maps of lymphoid-specific DHS, with regions of human-mouse sequence conservation at the *Ikzf1* locus, identified several *cis*-acting regulatory elements that are likely responsible for modulating Ikaros expression in this developmental system. Mapping of histone modification signatures and the preinitiation and elongation forms of RNApII at the *Ikzf1* locus in thymocytes has provided further insight into the location of promoter and enhancer elements, residing in the vicinity of conserved lymphoid-specific DHS clusters.[23,24,32-34] The promoter-demarcating H3K4me3 and H3K9Ac were highly enriched over the previously characterized *Ikzf1* lympho-myeloid promoter B. On the other hand, a restricted enrichment of H3K4me2, H3K27Ac, and the preinitiation form of RNApII over the intronic *Ikzf1* DHS clusters was consistent with previous reports of their marking active and poised enhancers.[23,24,32-34]

Because the ultimate proof of enhancer activity is the ability to stimulate expression from the gene's promoter, the ability of putative enhancers was tested on the *Ikzf1* lympho-myeloid promoter in vivo. The *Ikzf1* enhancer regions were evaluated for their ability to counteract transcriptionally repressive chromatin, to increase transcriptional rate, and to promote cell type–specific expression of a transgenic reporter during lymphoid and myeloid lineage differentiation. Six of the 7 *Ikzf1* conserved regions showed enhancer activities in B cells and myeloid cells (Figure 7A,C). However, only when these enhancers were combined into 1 regulatory unit could they function as an locus control region (LCR) and counteract all restrictive chromatin effects. A similar combinatorial action of distantly located regulatory elements functioning together as an LCR has been described for the λ5 and the V-preB.[35]

Outside the hemo-lymphoid system, *Ikzf1* is expressed in neural precursors of the cortex. In isolation, none of the *Ikzf1*enhancers were capable of brain-specific reporter expression, but when combined into a single regulatory unit with LCR activity, expression in the appropriate brain cortical region was detected.

Ikzf1 is expressed at high levels in differentiating thymocytes, where it is required for their normal maturation. Loss of Ikaros in thymocytes causes their aberrant expansion and transformation.[9,10,17,25,26] The previously identified *Ikzf1* promoter is active only in B cells and myeloid cells, indicating that additional transcriptional factor input from an enhancer is needed for T cell–specific expression. Two of the 7 enhancers (D and H) were capable of supporting expression in T cells. The rest of the *Ikzf1* enhancers did not confer additional cell type specificity to the *Ikzf1* promoter, although they greatly increased promoter activity in B cells and myeloid cells (Figure 7A,C).

In the developing embryo, *Ikzf1* expression is first detected in the hemangioblast region and in HSC-enriched compartments, albeit at lower levels compared with fetal sites harboring committed lymphocyte precursors.[27] During transition from an MPP to an LMPP,[3-5] *Ikzf1* expression is upregulated. The LMPP is the first restriction point downstream of the HSC, where upregulation of lymphoid-specific genes and downregulation of stem cell– and erythroid- but not myeloid-specific genes is occurring in an Ikaros-dependent fashion.[4,6] Notably, the *Ikzf1* enhancer D was the only *Ikzf1* regulatory region responsible for augmenting reporter expression in the LMPP (Figure 7B), suggesting that transcriptional inputs from its elements are key to the increase in Ikaros expression and lymphoid lineage specification.[4,6]

The importance of the *Ikzf1* enhancer D was further established in the context of the *Ikzf1* locus by its deletion from an *Ikzf1* BAC reporter. The enhancer D–deleted BAC reporter exhibited a highly variegated expression compared with the parental reporter. Two highly conserved sequence clusters were mapped at the 5′ and 3′ end of enhancer D (DI and DIII, respectively). Notably, the LMPP and T-cell specificity of enhancer D was ascribed to DI, whereas both clusters supported permissive chromatin.

Insight into the transcriptional networks responsible for inducing *Ikzf1* expression at the HSC and the LMPP and during T-cell differentiation was obtained by motif analyses for transcription factor binding sites at key *Ikzf1* enhancers and chromatin enrichment of putative transcriptional regulators at the *Ikzf1* locus. Both enhancers D and H contained sites for transcription factors highly expressed in T- and B-cell precursors, such as TCF-1, Runx1, HEB (E-Box), Ikaros, Ets1, and c-Myc, that play a determining role in lymphocyte differentiation. Sites for factors expressed early in lymphocyte development, such as HoxA9, E2A, Satb1, and Mef2c, were present only at enhancer D, underscoring its unique activation and function among *Ikzf1* enhancers at the onset of lymphopoiesis (Figure 7B-C). Further studies on these regulators with respect to their unique or redundant contribution to *Ikzf1* regulation will further establish their importance and position in the regulatory network that underscores lymphoid lineage differentiation.

Inactivating deletions and mutations in the *IKZF1* locus have been reported as poor prognostic indicators in pre–B-cell progenitor acute lymphoblastic leukemia and early T-cell progenitor acute lymphoblastic leukemia in humans and mice.[12,13,15,36] Large deletions at the 7p

arm resulting in the loss of the whole *IKZF1* allele, as well as smaller deletions between exons 4 to 7 that produce dominant-negative IKAROS protein isoforms or between exons 2 to 7 that have a potential for generating either a null or dominant negative allele, have been reported. Finally, point mutations that abolish the DNA binding activity of IKAROS and also generate dominant negative IKAROS protein isoforms have been reported in association with acute lymphoblastic leukemias.[13,37-39] Mutations in the *IKZF1* locus occurring in the highly conserved *Ikzf1* regulatory regions may interfere with Ikaros expression and provide a mechanism of leukemia development.

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Authorship

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References

1. Georgopoulos K. Transcription factors required for lymphoid lineage commitment. *Curr Opin Immunol.* 1997;9(2):222-227.
2. Cobb BS, Smale ST. Ikaros-family proteins: in search of molecular functions during lymphocyte development. *Curr Top Microbiol Immunol.* 2005; 290:29-47.
3. Adolfsson J, Månsson R, Buza-Vidas N, et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell.* 2005;121(2):295-306.
4. Yoshida T, Ng SY, Zuniga-Pflucker JC, Georgopoulos K. Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol.* 2006;7(4):382-391.
5. Lai AY, Kondo M. Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med.* 2006; 203(8):1867-1873.
6. Ng SY, Yoshida T, Zhang J, Georgopoulos K. Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity.* 2009;30(4):493-507.
7. Kelley CM, Ikeda T, Koipally J, et al. Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol.* 1998;8(9):508-515.
8. Georgopoulos K, Bigby M, Wang JH, et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell.* 1994;79(1):143-156.
9. Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell.* 1995;83(2):289-299.
10. Gómez-del Arco P, Kashiwagi M, Jackson AF, et al. Alternative promoter usage at the Notch1 locus supports ligand-independent signaling in T cell development and leukemogenesis. *Immunity.* 2010;33(5):685-698.
11. Jeannet R, Mastio J, Macias-Garcia A, et al. Oncogenic activation of the Notch1 gene by deletion of its promoter in Ikaros-deficient T-ALL. *Blood.* 2010;116(25):5443-5454.
12. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature.* 2007; 446(7137):758-764.
13. Mullighan CG, Miller CB, Radtke I, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature.* 2008;453(7191): 110-114.
14. Mullighan CG, Su X, Zhang J, et al; Children's Oncology Group. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med.* 2009;360(5):470-480.
15. Georgopoulos K. Acute lymphoblastic leukemia—on the wings of IKAROS. *N Engl J Med.* 2009;360(5):524-526.
16. Kaufmann C, Yoshida T, Perotti EA, Landhuis E, Wu P, Georgopoulos K. A complex network of regulatory elements in Ikaros and their activity during hemo-lymphopoiesis. *EMBO J.* 2003; 22(9):2211-2223.
17. Zhang J, Jackson AF, Naito T, et al. Harnessing of the nucleosome-remodeling-deacetylase complex controls lymphocyte development and prevents leukemogenesis. *Nat Immunol.* 2012; 13(1):86-94.
18. Li L, Zhang JA, Dose M, et al. A far downstream enhancer for murine Bcl11b controls its T-cell specific expression. *Blood.* 2013;122(6):902-911.
19. Yu M, Mazor T, Huang H, et al. Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. *Mol Cell.* 2012; 45(3):330-343.
20. Consortium EP; ENCODE Project Consortium. A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol.* 2011;9(4):e1001046.
21. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009;10(3):R25.
22. Zhang Y, Liu T, Meyer CA, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 2008;9(9):R137.
23. Ernst J, Kheradpour P, Mikkelsen TS, et al. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature.* 2011; 473(7345):43-49.
24. Kim TK, Hemberg M, Gray JM, et al. Widespread transcription at neuronal activity-regulated enhancers. *Nature.* 2010;465(7295):182-187.
25. Avitahl N, Winandy S, Friedrich C, Jones B, Ge Y, Georgopoulos K. Ikaros sets thresholds for T cell activation and regulates chromosome propagation. *Immunity.* 1999;10(3):333-343.
26. Winandy S, Wu L, Wang JH, Georgopoulos K. Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. *J Exp Med.* 1999;190(8):1039-1048.
27. Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science.* 1992;258(5083):808-812.
28. Agoston DV, Szemes M, Dobi A, et al. Ikaros is expressed in developing striatal neurons and involved in enkephalinergic differentiation. *J Neurochem.* 2007;102(6):1805-1816.
29. Martín-Ibáñez R, Crespo E, Urbán N, et al. Ikaros-1 couples cell cycle arrest of late striatal precursors with neurogenesis of enkephalinergic neurons. *J Comp Neurol.* 2010;518(3):329-351.
30. Elliott J, Jolicoeur C, Ramamurthy V, Cayouette M. Ikaros confers early temporal competence to mouse retinal progenitor cells. *Neuron.* 2008; 60(1):26-39.
31. Germar K, Dose M, Konstantinou T, et al. T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. *Proc Natl Acad Sci USA.* 2011;108(50):20060-20065.
32. De Santa F, Barozzi I, Mietton F, et al. A large fraction of extragenic RNA pol II transcription sites overlap enhancers. *PLoS Biol.* 2010;8(5): e1000384.
33. Pekowska A, Benoukraf T, Zacarias-Cabeza J, et al. H3K4 tri-methylation provides an epigenetic signature of active enhancers. *EMBO J.* 2011; 30(20):4198-4210.

34. Koch F, Fenouil R, Gut M, et al. Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. *Nat Struct Mol Biol.* 2011;18(8):956-963.

35. Sabbattini P, Dillon N. The lambda5-VpreB1 locus—a model system for studying gene regulation during early B cell development. *Semin Immunol.* 2005;17(2):121-127.

36. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature.* 2012;481(7380):157-163.

37. Iacobucci I, Iraci N, Messina M, et al. IKAROS deletions dictate a unique gene expression signature in patients with adult B-cell acute lymphoblastic leukemia. *PLoS ONE.* 2012;7(7): e40934.

38. Dupuis A, Gaub MP, Legrain M, et al. Biclonal and biallelic deletions occur in 20% of B-ALL cases with IKZF1 mutations. *Leukemia.* 2013;27(2): 503-507.

39. Kastner P, Dupuis A, Gaub MP, Herbrecht R, Lutz P, Chan S. Function of Ikaros as a tumor suppressor in B cell acute lymphoblastic leukemia. *Am J Blood Res.* 2013;3(1):1-13.