

Ikaros controls isotype selection during immunoglobulin class switch recombination

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Class switch recombination (CSR) allows the humoral immune response to exploit different effector pathways through specific secondary antibody isotypes. However, the molecular mechanisms and factors that control immunoglobulin (Ig) isotype choice for CSR are unclear. We report that deficiency for the Ikaros transcription factor results in increased and ectopic CSR to IgG_{2b} and IgG_{2a}, and reduced CSR to all other isotypes, regardless of stimulation. Ikaros suppresses active chromatin marks, transcription, and activation-induced cytidine deaminase (AID) accessibility at the γ 2b and γ 2a genes to inhibit class switching to these isotypes. Further, Ikaros directly regulates isotype gene transcription as it directly binds the *Igh* 3' enhancer and interacts with isotype gene promoters. Finally, Ikaros-mediated repression of γ 2b and γ 2a transcription promotes switching to other isotype genes by allowing them to compete for AID-mediated recombination at the single-cell level. Thus, our results reveal transcriptional competition between constant region genes in individual cells to be a critical and general mechanism for isotype specification during CSR. We show that Ikaros is a master regulator of this competition.

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Abbreviations used: 3C, chromosome conformation capture; AcH3, histone H3 acetylation; AID, activation-induced cytidine deaminase; ChIP, chromatin immunoprecipitation; CSR, class switch recombination; DSB, double-stranded DNA break; GLT, germline transcript; HDAC, histone deacetylase; HS, hypersensitive; I, intronic; NGFR, nerve growth factor receptor; qPCR, quantitative PCR; S, switch; SC, single cell.

Class switch recombination (CSR) diversifies the humoral immune response by joining a single antibody variable region gene with different constant region (C_H) genes responsible for unique effector functions (1). This is crucial for establishing immunity, as patients selectively deficient in CSR suffer from recurrent and severe infections (2). CSR occurs between repetitive but nonhomologous DNA sequences called switch (S) regions, which are located upstream of each C_H gene (except δ). CSR requires the expression of activation-induced cytidine deaminase (AID) (3, 4), an enzyme that is thought to directly deaminate single-stranded DNA (5, 6), though this mechanism is still under debate (7). DNA lesions induced by AID are processed to generate double-stranded DNA breaks (DSBs), which activate DNA damage response proteins to promote efficient long-range recombination (8). DSBs in S μ and downstream S regions are

ultimately joined through end joining mechanisms, allowing the expression of a new antibody isotype (1).

CSR requires transcription and is targeted to individual constant region genes by the selective activation of isotype-specific intronic (I) promoters in response to antigen, cytokine, and co-stimulatory signals (9). This “germline transcription” begins at I exons and proceeds through adjacent S regions and C_H genes, giving rise to noncoding germline transcripts (GLTs). Transcription is thought to initiate CSR by promoting S region accessibility and exposing single-stranded DNA to AID (1). Indeed, CSR is abrogated by I promoter deletions (10, 11) and is restored by their replacement with heterologous promoters (12, 13). These latter studies also demonstrated that constitutively transcribed S regions are ectopically targeted for CSR, highlighting the role of S region transcription in

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isotype selection. However, the mechanisms establishing this targeting are not completely understood, and it is unclear how individual cells select between simultaneously transcribed S regions for CSR.

Germline transcription is regulated by an enhancer at the 3' end of the *Igh* locus and by chromatin modifications. The 30-kb *Igh* 3' enhancer lies downstream of C α and contains four DNase hypersensitive (HS) regions: HS3a, HS1,2, HS3b, and HS4. Disruption of the enhancer reduces transcription and CSR to all isotypes, with γ 3, γ 2b, and γ 2a most drastically affected (14, 15). As the 3' enhancer is distant from I promoters (up to 110 kb), transcriptional control is believed to occur through promoter–enhancer looping (16). In addition, histone modifications, such as histone H3 acetylation (AcH3) at I exons and S regions, are tightly correlated with GLT induction, indicating that they may regulate germline transcription (17, 18). Nonetheless, the molecular mechanisms and factors controlling S region transcription and isotype specification during CSR remain largely undefined.

The Ikaros zinc finger transcription factor plays important roles in B cells. Ikaros is required for B cell specification (19, 20) and differentiation (20–22), as well as allelic exclusion at the *Igk* locus (23, 24). We have studied Ikaros function in the B cell lineage using mice bearing a hypomorphic mutation in the *ikzf1* (Ikaros) locus ($Ik^{L/L}$). $Ik^{L/L}$ mice contain a LacZ reporter knocked into *ikzf1* exon 2, resulting in the production of low levels of functional, but truncated, Ikaros proteins (\sim 10% of WT) in hematopoietic cells (21). Unlike Ikaros-null mice (19), $Ik^{L/L}$ mice develop relatively normal numbers of mature, polyclonal B cells (21). Interestingly, $Ik^{L/L}$ mice exhibit abnormal serum antibody titers, characterized by striking $>$ 50% reductions in IgG₃ and IgG₁, and $>$ 50% increases in IgG_{2b} and IgG_{2a} (21). This intriguing observation led us to hypothesize that Ikaros plays a role in isotype selection. In this paper, we report that Ikaros is indeed a central regulator of *Igh* locus transcription and isotype specification during CSR.

RESULTS

Ikaros deficiency skews CSR to IgG_{2b} and IgG_{2a}

To determine if Ikaros regulates CSR, switching to all isotypes was assessed in purified WT and $Ik^{L/L}$ splenic B220⁺ B cells using a battery of in vitro culture conditions. CSR was measured by flow cytometry (FACS) for surface Ig isotype expression after 3–4 d in culture. After LPS stimulation, WT cells switched only to IgG₃ and IgG_{2b}, as expected (Fig. 1 A and Fig. S1 A). In contrast, IgG_{2b}⁺ cells were 3.6-fold more frequent in $Ik^{L/L}$ cultures, and IgG_{2a}⁺ cells were also detected, whereas IgG₃⁺ cells were reduced (23% of WT; Fig. 1 A and Fig. S1 A). After LPS + IFN- γ stimulation, both genotypes switched to the same isotypes, but $Ik^{L/L}$ cultures produced more IgG_{2b}⁺ and IgG_{2a}⁺ cells (up 2.3- and 2.7-fold, respectively, vs. WT) and fewer IgG₃⁺ cells (24% of WT; Fig. 1 B and Fig. S1 B). After LPS + IL-4 stimulation, WT cells switched to IgG₁ (Fig. 1 C and Fig. S1 C) and IgE (as determined by RT-quantitative PCR [qPCR] of ϵ postswitch transcripts; Fig. 2 A) (25), as expected. In contrast, $Ik^{L/L}$ cells switched

ectopically to IgG_{2b}, IgG_{2a}, and IgG₃, produced fewer IgG₁⁺ cells than WT (WT, $30.1 \pm 1.63\%$; $Ik^{L/L}$, $26.2 \pm 1.57\%$), and exhibited a 54% reduction in ϵ postswitch transcripts, indicating fewer IgE⁺ cells (Fig. 1 C, Fig. 2 A, and Fig. S1). After LPS + IL-5 + TGF- β stimulation, WT cells switched to IgG₃, IgG_{2b}, and IgA, whereas $Ik^{L/L}$ cultures generated 2.9-fold more IgG_{2b}⁺ cells, unexpected IgG_{2a}⁺ cells, and fewer IgG₃⁺ and IgA⁺ cells (32 and 52% of WT, respectively; Fig. 1 D and Fig. S1 D). In all of the these conditions, the fraction of switched cells (e.g., IgG⁺ and IgA⁺) in Ikaros-deficient cultures increased 20–120% versus WT, suggesting a general increase in switching capacity (Fig. 1). Finally, we confirmed these results by ELISA (Fig. S2), demonstrating that isotype expression is significantly skewed toward IgG_{2b} and IgG_{2a} in activated Ikaros-deficient B cells.

Because CSR requires proliferation (26) and $Ik^{L/L}$ B cells hyperproliferate at low stimuli concentrations and cell densities (21), overall increases in switched cells and/or specific increases in IgG_{2b}⁺ and IgG_{2a}⁺ cells could be caused by increased cell divisions in $Ik^{L/L}$ cultures. To evaluate this, CFSE dilution was used to track cell division, and the percentage of IgG/A⁺ WT and $Ik^{L/L}$ cells in each generation was compared for the described conditions. Each generation of $Ik^{L/L}$ cells exhibited increases in IgG_{2b}⁺ and IgG_{2a}⁺ cells, as well as increased overall switching after all stimulations when compared with WT; increases in IgG₃⁺ cells were also detected after LPS + IL-4 stimulation (Fig. S3). Further, $Ik^{L/L}$ cells exhibited reduced CSR to IgG₃ and IgA at every division analyzed and to IgG₁ after the third division in conditions that induce WT cells to switch to these isotypes (LPS, LPS + IFN- γ , and LPS + IL-5 + TGF- β for IgG₃, LPS + IL-4 for IgG₁, and LPS + IL-5 + TGF- β for IgA; Fig. S3). These results indicate that significant, proliferation-independent isotype expression abnormalities exist in $Ik^{L/L}$ B cells after CSR-inducing stimulation.

It was also possible that the increased IgG_{2b}⁺ and IgG_{2a}⁺ populations in $Ik^{L/L}$ cultures could be caused by the expansion of autoreactive cells that had previously switched in vivo, rather than specific CSR defects, considering the lower activation thresholds of $Ik^{L/L}$ B cells (21) and the production of autoantibodies in transgenic mice expressing a B cell-restricted dominant-negative Ikaros isoform (27). Thus, isotype expression was analyzed using whole and bona fide unswitched B cells (B220⁺, and CD43⁻IgG/A⁻ and CD43⁻IgM⁺, respectively) that were stimulated with LPS, LPS + IFN- γ , and LPS + IL-4. Regardless of purification strategy, $Ik^{L/L}$ cultures exhibited consistent and significant increases in IgG_{2b}⁺ and IgG_{2a}⁺ (and IgG₃⁺ with LPS + IL-4) cells compared with WT cultures (Fig. S4). Further, there were no significant differences in IgG_{2b} or IgG_{2a} expression between B220⁺, CD43⁻IgG/A⁻, and CD43⁻IgM⁺ $Ik^{L/L}$ cultures (Fig. S4). Thus, the aberrant isotype expression by Ikaros-deficient B cells results from defects in CSR and not from the expansion of in vivo-switched cells.

In summary, $Ik^{L/L}$ cells (a) are more likely to undergo CSR, (b) display enhanced and ectopic CSR to IgG_{2b} and IgG_{2a} regardless of stimulation, and (c) show reduced CSR to all other isotypes (except for ectopic CSR to IgG₃ after LPS + IL-4).

Therefore, Ikaros deficiency results in a B cell-intrinsic defect in isotype specification during CSR.

Ikaros function is required during CSR

Because Ikaros is expressed throughout B cell development and controls processes such as V(D)J recombination, which

may affect the B cell repertoire (19, 20), we asked if Ikaros is required in mature B cells during CSR. WT B cells were stimulated for 24 h with LPS to induce CSR, and were then transduced with retroviruses encoding the dominant-negative Ikaros 6 (Ik6) isoform and a truncated human nerve growth factor receptor (NGFR) reporter. The Ik6 isoform lacks

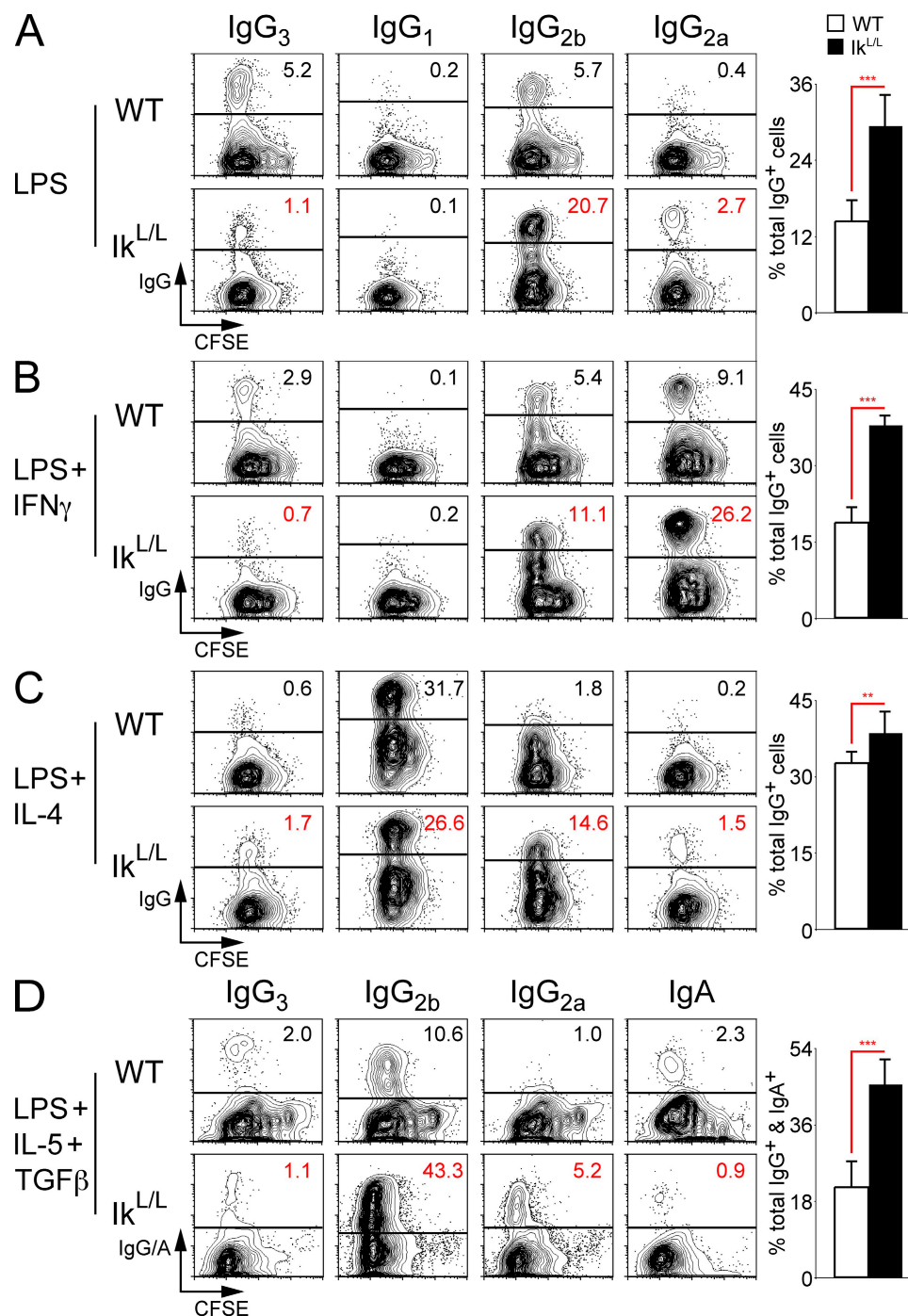


Figure 1. CSR is skewed toward IgG_{2b} and IgG_{2a} in Ik^{L/L} B cells. CFSE-labeled B220⁺ WT and Ik^{L/L} B cells were stimulated for 72 h with (A) LPS, (B) LPS + IFN- γ , or (C) LPS + IL-4. (D) 24 h with LPS was followed by 72 h with LPS + IL-5 + TGF- β . CSR was analyzed by FACS, and percentages of Ig⁺ cells are indicated. The data are representative of more than four independent experiments. Bar graphs represent mean percentages plus SD of switched cells in each condition for four (D) or five (A–C) experiments. Significance was determined by a two-tailed *t* test assuming unequal variance (***, *P* < 0.005; **, *P* < 0.02).

DNA binding domain zinc fingers and, upon dimerization, inhibits DNA binding by functional Ikaros isoforms (28). 72 h after infection, switching and NGFR expression were analyzed by FACS. WT cells transduced with control virus (NGFR^{hi}) switched exclusively to IgG₃ and IgG_{2b}, as did uninfected cells (NGFR⁻) from WT cultures transduced with Ik6-NGFR (Fig. 3). Ik6-NGFR^{hi} cells, however, exhibited a threefold increase in IgG_{2b}⁺ cells, ectopic CSR to IgG_{2a}, 30–50% fewer IgG₃⁺ cells, and more switched cells (Fig. 3 and Fig. S5 A). Thus, inhibiting Ikaros function in mature WT B cells during CSR recapitulated the Ik^{L/L} switching phenotype. Similarly, transduction of Ik^{L/L} cells with the full-length Ik1 isoform rescued Ik^{L/L} switching defects (Fig. S5, B and C). Importantly, equally effective rescue was achieved with the truncated Ik1 isoform that lacks exon 2–derived protein sequences and is expressed at low levels in Ik^{L/L} cells, indicating that these truncated Ikaros proteins function like full-length Ikaros during CSR (Fig. S5, B and C) (21). Collectively these results show that Ikaros regulates CSR specificity and efficiency in mature B cells.

AID expression is normal in Ik^{L/L} B cells

AID protein levels correlate with *aicda* transcript levels and switching efficiency (29–31). Thus, if *aicda*/AID expression were increased in Ikaros-deficient B cells, it could account for increased switching. *Aicda* expression was examined by RT-qPCR in WT and Ik^{L/L} B cells, either freshly isolated or stimulated for 48 h with LPS, LPS + IFN- γ , or LPS + IL-4. Stimulation of WT and Ik^{L/L} B cells induced *aicda* expression >48-fold, and there was no significant difference in *aicda* levels between Ik^{L/L} and WT, though expression was slightly higher

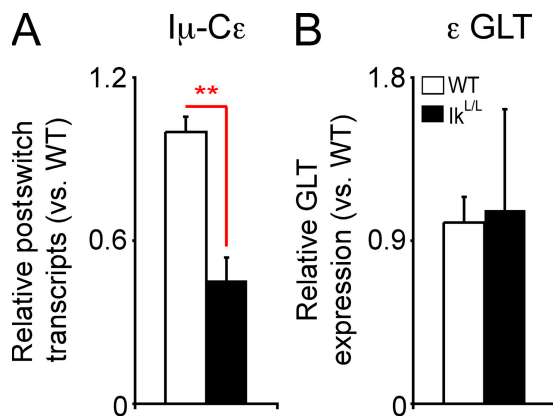


Figure 2. Reduced IgE switching in Ik^{L/L} B cells despite normal ϵ GLT expression. (A) I μ -C ϵ transcript expression in B220⁺ WT and Ik^{L/L} B cells after 72 h of stimulation with LPS + IL-4 was analyzed by RT-qPCR. The I μ promoter is equally active in WT and Ik^{L/L} cells (Fig. 4, A–D), indicating that I μ -C ϵ transcripts can be faithfully compared between genotypes to quantify IgE-switched cells. (B) RT-qPCR for ϵ GLT expression after 48 h of LPS + IL-4 stimulation. *Ig β* was used as a loading control and all values were normalized to WT. Significance was determined by a two-tailed *t* test assuming unequal variance (**, *P* < 0.02). Graphs represent means plus SD of two (A) or three (B) independent experiments. Neither I μ -C ϵ transcripts nor ϵ GLTs were detected in other conditions (not depicted).

in mutant cells (Fig. S6). Further, multiplex single-cell (SC)–RT-PCR analysis revealed similar percentages of *aicda*⁺ cells in both genotypes after LPS (WT, 71%; Ik^{L/L}, 67%) and LPS + IL-4 (WT, 65%; Ik^{L/L}, 62%) stimulation, suggesting similar levels of *aicda* transcripts per cell (Table S1; see Fig. 9, A and B). Thus, higher CSR efficiency in Ik^{L/L} cells is not caused by increased *aicda* expression.

Ikaros deficiency results in deregulated S region transcription

To determine if Ikaros controls CSR specificity by regulating S region transcription, we measured GLT levels for μ , γ 3, γ 1, γ 2b, γ 2a, and ϵ in WT and Ik^{L/L} B cells by RT-qPCR. Freshly isolated Ik^{L/L} cells exhibited three- to sixfold increases in γ 3, γ 2b, and γ 2a GLT expression at the population level (Fig. 4 A). Further, SC-RT-PCR revealed that ~22 and ~6% of Ik^{L/L} cells expressed γ 3 and γ 2b GLTs, respectively, whereas virtually no WT cells expressed either GLT, suggesting that Ikaros represses the transcription of a subset of S regions in the absence of ex vivo stimulation (Fig. S7 and Table S1). After 48 h with LPS, LPS + IFN- γ , or LPS + IL-4, WT B cells specifically induced GLTs for the isotypes to which they switched (Fig. 1, A–C; and Fig. 4, B–D). Ik^{L/L} cells, however, expressed markedly different patterns of GLTs, and only derepressed GLTs correlated well with CSR. Strikingly, γ 2b and γ 2a GLTs were either overexpressed (>3.5-fold) or expressed ectopically (γ 2b with LPS + IL-4, and γ 2a with LPS and LPS + IL-4) in Ik^{L/L} cells, and this expression correlated with increased or ectopic CSR to these isotypes (Fig. 1, A–C; Fig. 4, B–D; and Fig. S8). Similarly, γ 3 GLTs were expressed ectopically with LPS + IL-4 stimulation, which correlated with ectopic IgG₃ switching (Fig. 1 C and Fig. 4 D). In contrast, μ GLTs were expressed similarly in both genotypes, and surprisingly, despite expressing WT levels of γ 3 (with LPS and LPS + IFN- γ), γ 1 and ϵ GLTs, Ik^{L/L} cells switched less efficiently to these isotypes (Fig. 1, A–C; Fig. 2; and Fig. 4, B–D). Collectively, these results suggest that Ikaros represses the transcription of S γ 3, S γ 2b, and S γ 2a in resting and stimulated cells, and that increased germline transcription of S γ 2b and S γ 2a (and S γ 3 after LPS + IL-4) in Ikaros-deficient cells skews isotype selection during CSR.

Derepression of S region transcription in Ik^{L/L} cells results in increased AID-dependent histone modifications

If enhanced S γ 2b and S γ 2a transcription skews switching in Ik^{L/L} cells, it would be reflected in increased AID accessibility and activity in these S regions, as AID-induced DSBs are necessary intermediates for CSR (32). To evaluate AID accessibility, we measured AcH4, which is induced in response to DSBs (33, 34) and is up-regulated in an AID-dependent manner at S regions during CSR (18). S region AcH4 levels were analyzed in both freshly isolated and stimulated (48 h) WT and Ik^{L/L} cells by chromatin immunoprecipitation (ChIP)–qPCR using anti-AcH4 antibodies. S region AcH4 levels were low in unstimulated B cells of both genotypes, as expected (Fig. 5 A). In WT cells, LPS and LPS + IFN- γ

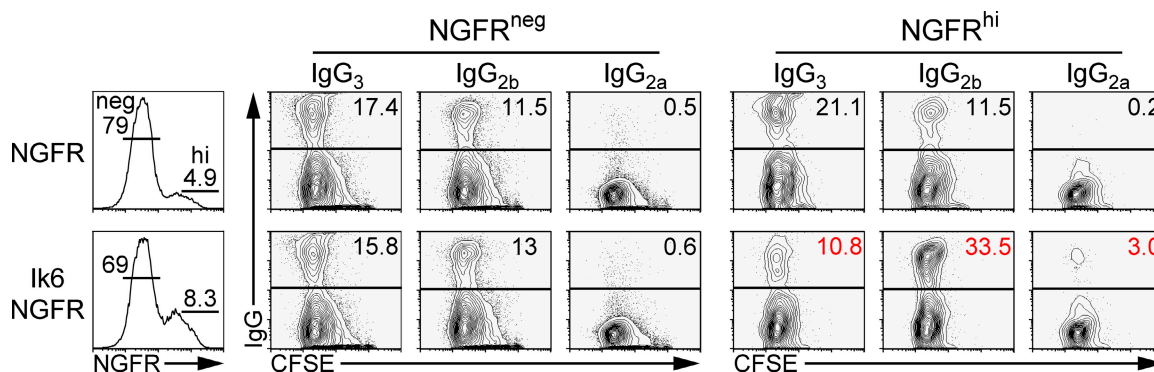


Figure 3. Ikaros regulates CSR in mature B cells. CFSE-stained B220⁺ WT B cells were activated for 24 h with LPS to induce CSR, infected with retroviruses encoding the NGFR reporter alone or with the dominant-negative Ik6 isoform (Ik6-NGFR), and stimulated for another 72 h with LPS. Ig and NGFR expression were analyzed by FACS. Numbers represent percentages of NGFR⁻, NGFR⁺, or IgG⁺ cells. The data are representative of four independent experiments (Fig. S5 A provides a statistical analysis).

induced H4 acetylation at $\gamma 3$ and $\gamma 2b$, and LPS + IFN- γ also induced AcH4 at $\gamma 2a$; LPS + IL-4 induced AcH4 exclusively at $\gamma 1$ (Fig. 5, B–D). Thus, AcH4 levels mirrored GLT and isotype expression in WT cells, confirming that AID-dependent chromatin modifications correlate with transcription and switching (Figs. 1, 4, and 5) (18).

In Ik^{L/L} cells, however, histone H4 was hyperacetylated at ectopically and overtranscribed S regions. AcH4 levels were consistently higher at $\gamma 2b$ and $\gamma 2a$ in stimulated Ik^{L/L} cells compared with WT, correlating with increased transcription and switching to these isotypes (Fig. 1, A–C; Fig. 4, B–D; and Fig. 5, B–D). Similarly, ectopic AcH4 at $\gamma 3$ in LPS +

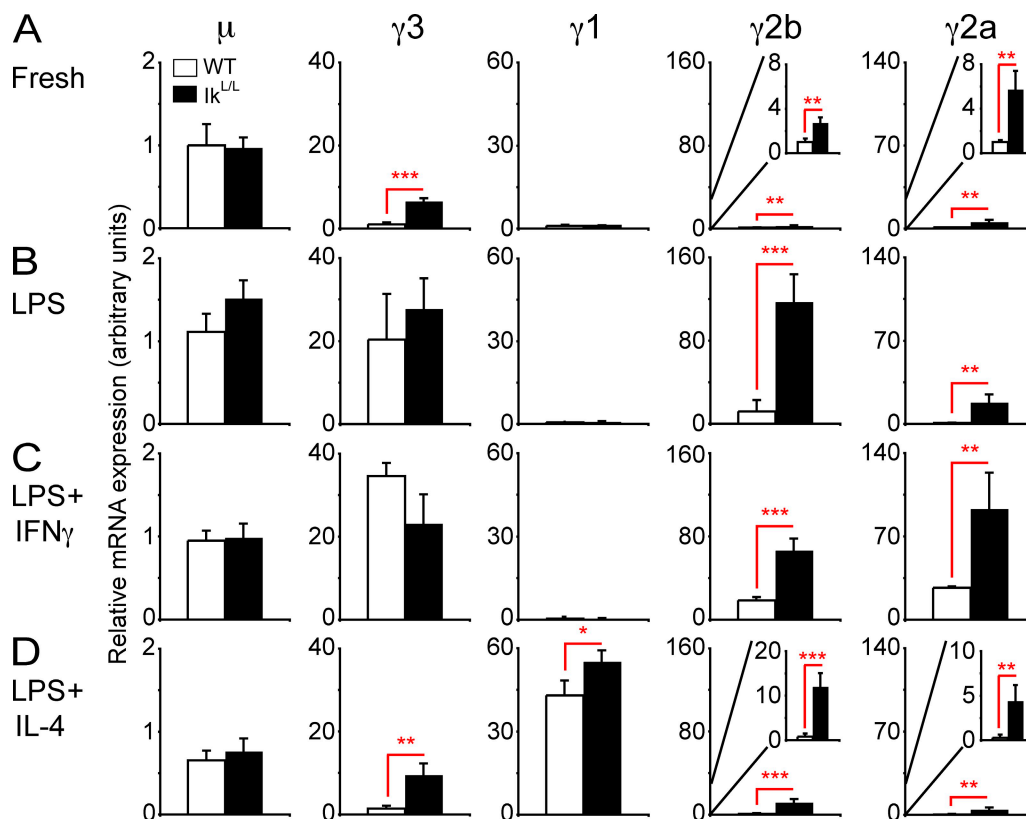


Figure 4. Deregulated expression of $\gamma 3$, $\gamma 2b$, and $\gamma 2a$ GLTs in Ik^{L/L} B cells. GLTs expressed by B220⁺ WT and Ik^{L/L} B cells that were (A) freshly isolated or stimulated for 48 h with (B) LPS, (C) LPS + IFN- γ , or (D) LPS + IL-4 were analyzed by qPCR. GLTs were normalized to *Ig β* levels and all values are represented relative to those of unstimulated WT B cells. Graphs represent means plus SD of three independent experiments. Significance was determined by a two-tailed *t* test assuming unequal variance (***, $P < 0.005$; **, $P < 0.02$; *, $P < 0.05$).

IL-4-stimulated $Ik^{L/L}$ cells mirrored ectopic $\gamma 3$ transcription and switching (Fig. 1 C, Fig. 4 D, and Fig. 5 D). In contrast, normal $\gamma 3$ GLT expression in LPS- and LPS + IFN- γ -stimulated $Ik^{L/L}$ cells induced 25–40% lower AcH4 levels at $S\gamma 3$ in comparison with WT, suggesting reduced AID-dependent activity (Fig. 4, B and C; and Fig. 5, B and C). Finally, similar $\gamma 1$ GLT levels in LPS + IL-4-stimulated $Ik^{L/L}$ and WT cells resulted in comparable induction of AcH4 at $S\gamma 1$ in both

genotypes (Fig. 4 D and Fig. 5 D). Thus, increased transcription of $S\gamma 2b$ and $S\gamma 2a$ (and $S\gamma 3$ after LPS + IL-4) correlates with increased AID-dependent H4 acetylation, suggesting that abnormal germline transcription in $Ik^{L/L}$ cells increases AID accessibility at $S\gamma 2b$ and $S\gamma 2a$ (and $S\gamma 3$) to skew CSR.

Ikaros does not repress germline transcription by regulating promoter–enhancer interactions in the *Igh* locus

Germline transcription of $S\gamma 3$, $S\gamma 2b$, and $S\gamma 2a$ requires the *Igh* 3' enhancer (14, 15), and Ikaros has been reported to regulate long-range promoter–enhancer looping at the β -globin locus (35). Therefore, we asked if Ikaros regulates germline transcription by controlling interactions between the 3' enhancer and C_H gene promoters. *Igh* locus promoter–enhancer interactions were assayed in WT and $Ik^{L/L}$ B cells by chromosome conformation capture (3C) to measure interactions between distal chromosome regions (36). In these experiments, interacting chromatin was cross-linked with formaldehyde, digested with a restriction enzyme, and subjected to intramolecular DNA ligation and qPCR amplification of ligation products (Fig. S9 shows digestion and PCR controls). We examined interactions between a HindIII fragment containing the HS1,2 regulatory site and fragments covering the μ enhancer, downstream C_H gene promoters, HS3a, and HS3b/4, as well as I regions not expected to participate in the regulation of germline transcription. Although HS1,2 was chosen as a reference point, patterns of promoter–enhancer cross-linking were similar, though weaker, using a 3' enhancer fragment containing HS3b/4 (unpublished data). WT and $Ik^{L/L}$ B cells were compared, either before or after 36 h of stimulation, when germline transcription was efficiently induced, but CSR was barely detectable by FACS (unpublished data). B cell-specific interactions were identified by comparison with PMA-stimulated T cells in which the *Igh* locus is silent.

In freshly isolated B cells, but not T cells, HS1,2 was frequently cross-linked to a fragment containing the μ enhancer, as previously reported (Fig. 6 A) (16). This interaction was reduced but still present in $Ik^{L/L}$ B cells, suggesting that Ikaros contributes to μ -3' enhancer interactions (Fig. 6 A). Cross-linking between HS1,2 and $\gamma 3$ or $\gamma 2a$ in $Ik^{L/L}$ B cells was similar to WT, despite ectopic $\gamma 3$, $\gamma 2b$, and $\gamma 2a$ transcription; only HS1,2- $\gamma 2b$ interactions were increased in mutant cells (Fig. 4 A and Fig. 6 A). Thus no clear correlation was detected between C_H promoter–HS1,2 interactions and increased GLT expression in unstimulated $Ik^{L/L}$ cells.

Next, we asked if Ikaros deficiency affects promoter–enhancer interactions after stimulation. In LPS-induced WT cells, there were B cell-specific HS1,2- $\gamma 3$ and - $\gamma 2b$ cross-linking peaks, indicating HS1,2 interactions with these C_H genes during transcription and switching (Fig. 1 A, Fig. 4 B, and Fig. 6 B). In $Ik^{L/L}$ cells, HS1,2- $\gamma 2b$ and - $\gamma 2a$ cross-linking frequencies were similar to WT despite enhanced germline transcription of these genes (Fig. 4 B and Fig. 6 B); HS1,2- $\gamma 3$ interactions were also similar between genotypes. After LPS + IFN- γ stimulation, WT cells exhibited significant B cell-specific cross-linking between HS1,2 and $\gamma 2a$, as well as interaction peaks

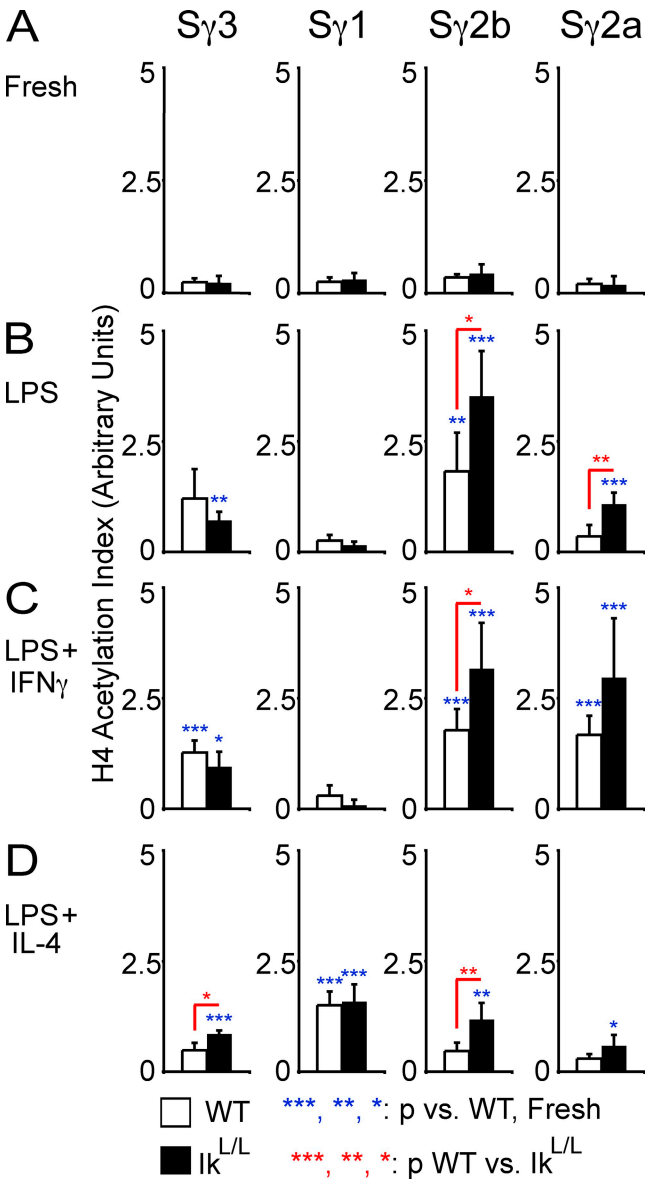


Figure 5. AcH4 correlates with transcriptional deregulation in $Ik^{L/L}$ B cells. WT and $Ik^{L/L}$ CD43⁻ B cells that were (A) freshly isolated or stimulated for 48 h with (B) LPS, (C) LPS + IFN- γ , or (D) LPS + IL-4 were subjected to ChIP with anti-AcH4 antibodies. Graphs represent the mean S region AcH4 enrichment indexes plus SD for three to four independent experiments. Significance was determined by a two-tailed *t* test assuming unequal variance (***, $P < 0.005$; **, $P < 0.02$; *, $P < 0.05$). AcH4 at $S\gamma 2a$ was consistently higher in $Ik^{L/L}$ versus WT samples after LPS + IFN- γ ($S\gamma 2a$ $Ik^{L/L}$ /WT ratio mean = 1.68; range = 1.21–2.13).

between HS1,2 and $\gamma 3$ and $\gamma 2b$, which correlated with transcription and switching for all three genes (Fig. 1 B, Fig. 4 C, and Fig. 6 C). HS1,2– $\gamma 2b$ and – $\gamma 2a$ interactions were nearly

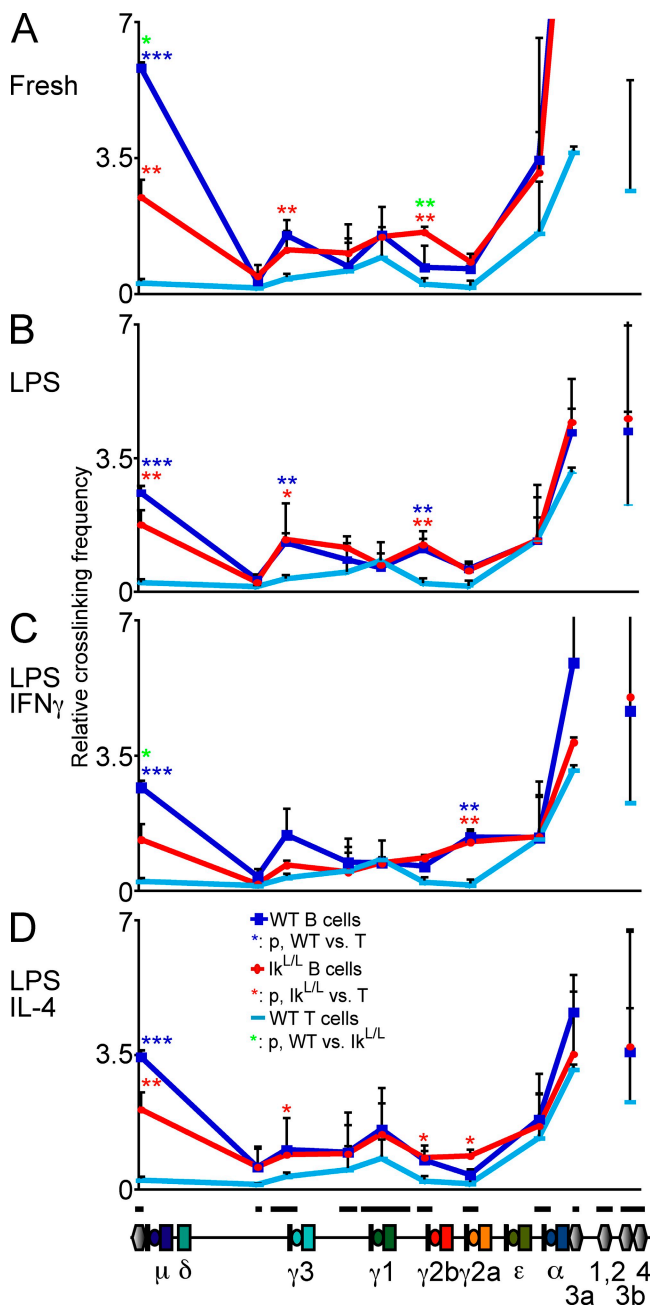


Figure 6. Ikaros does not regulate interactions between HS1,2 and C_H gene promoters. CD43⁻ WT and Ik^{L/L} B cells were analyzed by 3C, either (A) before or after 36 h with (B) LPS, (C) LPS + IFN- γ , or (D) LPS + IL-4. CD4⁺ T cells stimulated for 36 h with PMA served as a negative control. The y axis represents the relative cross-linking frequency between a HindIII fragment covering HS1,2 and the rest of the locus. Points represent means plus SD of two independent experiments. Significance was determined by a two-tailed *t* test assuming unequal variance (***, $P < 0.005$; **, $P < 0.02$; *, $P < 0.05$). In A, HS1,2–HS3a and –HS3b/4 cross-linking frequencies in B cells, though similar between genotypes, were too high to be shown.

identical in Ik^{L/L} cells, despite sharply increased transcription and switching mainly to $\gamma 2a$ and, to a lesser extent, $\gamma 2b$ and $\gamma 3$ (Fig. 1 B, Fig. 4 C, and Fig. 6 C). On the other hand, HS1,2– $\gamma 3$ interactions were somewhat, but not significantly reduced in Ik^{L/L} cells (despite normal transcription), indicating a possible role for Ikaros in HS1,2– $\gamma 3$ interactions after LPS + IFN- γ (Fig. 6 C). Finally, LPS + IL-4 induced consistently higher HS1,2– $\gamma 1$ interactions in WT and Ik^{L/L} B cells versus WT T cells, which correlated with transcription and switching at $\gamma 1$ in response to LPS + IL-4, as previously reported (Fig. 1 C, Fig. 4 D, and Fig. 6 D) (16). Importantly, ectopic transcription of $\gamma 3$, $\gamma 2b$, and $\gamma 2a$ in LPS + IL-4-stimulated Ik^{L/L} cells did not correlate with modified promoter–enhancer interactions, as there were no significant differences between WT and Ik^{L/L} cells, and only HS1,2– $\gamma 2a$ cross-linking was slightly increased in mutant populations (Fig. 4 D and Fig. 6 D). In summary, similar to previous reports, we found interactions between HS1,2 and C_H genes that were transcribed and targeted for CSR in WT cells (16). Further, there was no clear correlation between C_H promoter–HS1,2 interactions and defects in germline transcription. Therefore, deregulated S region transcription and aberrant switching in Ikaros-deficient B cells is not caused by altered interactions between the *Igh* 3' enhancer and the promoters of specific C_H genes.

Ikaros maintains repressive chromatin at the $\gamma 2b$ and $\gamma 2a$ genes

Ach3 is a hallmark of transcriptionally active chromatin (37) and correlates tightly with germline transcription (17, 18). Thus, we asked if Ikaros could regulate S region transcription by controlling H3 acetylation. Ach3 was examined at $C\gamma$ I exons and S regions in WT and Ik^{L/L} B cells by ChIP–qPCR, both before and after 48 h of stimulation. There were no significant differences in Ach3 levels between freshly isolated WT and Ik^{L/L} cells (Fig. 7 A). This suggests that Ach3 levels either do not contribute to GLT overexpression, or that our assay lacked the sensitivity required to detect differences in Ach3 associated with low levels of transcription (Fig. 4 A).

Next, we determined if Ach3 levels correlate with deregulated germline transcription in Ik^{L/L} B cells after stimulation. Stimulated WT cells induced H3 acetylation at the isotype genes they transcribed: I/S $\gamma 3$ and I/S $\gamma 2b$ with LPS and LPS + IFN- γ , I/S $\gamma 2a$ with LPS + IFN- γ , and I/S $\gamma 1$ with LPS + IL-4 (Fig. 4, B–D; and Fig. 7, B–D). Strikingly, in stimulated Ik^{L/L} cells, Ach3 levels at the $\gamma 2b$ and $\gamma 2a$ I exons and/or S regions were consistently higher than in WT cells, and correlated with increased and ectopic transcription (Fig. 4, B–D; and Fig. 7, B–D). Similarly, ectopic Ach3 at I/S $\gamma 3$ in LPS + IL-4-stimulated Ik^{L/L} cells correlated with ectopic transcription of that isotype (Fig. 4 D and Fig. 7 D). Normal levels of $\gamma 3$ (LPS and LPS + IFN- γ) and $\gamma 1$ (LPS + IL-4) transcription in Ik^{L/L} cells were associated with Ach3 levels at I/S $\gamma 3$ and I/S $\gamma 1$ that were equivalent to WT (Fig. 4, B–D; and Fig. 7, B–D). Importantly, Ach3 enrichment at the 3' enhancer was also similar between WT and Ik^{L/L} cells (unpublished data). Thus, deregulated transcription and Ach3 are tightly correlated in

$Ik^{L/L}$ cells, indicating that Ikaros promotes a repressive chromatin state (e.g., histone H3 hypoacetylation) at C_H promoters and genes during CSR.

Ikaros interacts directly with the *Igh* locus

We next asked if Ikaros could control chromatin remodeling and S region transcription through direct binding to *Igh* regulatory regions. We analyzed Ikaros binding to the *Igh* locus in freshly isolated and stimulated WT B cells by ChIP-qPCR using anti-Ikaros antibodies. We focused on regions in the *Igh* 3' enhancer and $C\gamma$ gene promoters, I exons and S regions, that contained Ikaros consensus sites identified with the TF-SEARCH algorithm (38).

Ikaros associated with the *Igh* locus in vivo in WT B cells (Fig. 8). In freshly isolated $CD43^-$ cells, Ikaros associated with $I\gamma 2b$ and HS1,2 in the 3' enhancer (Fig. 8 A). These associations were maintained after LPS stimulation (Fig. 8 B). When cells were stimulated with LPS + IFN- γ , Ikaros associated at low levels with $I\gamma 3$ and at higher levels with $I\gamma 2b$, $I\gamma 2a$, and HS1,2 (Fig. 8 C). Interestingly, this stimulation also

induced transcription at $I\gamma 3$, $I\gamma 2b$, and $I\gamma 2a$, indicating that Ikaros associates strongly with some I exons upon their transcriptional activation (Fig. 8 C). Similarly, after LPS + IL-4 stimulation, Ikaros associated with the transcribed $I\gamma 1$ exon (Fig. 4 D and Fig. 8 D). In addition, there was moderate Ikaros association with $I\gamma 2b$, whereas the HS1,2 peak remained high (Fig. 8 D). Thus, Ikaros strongly associates with HS1,2 regardless of stimulation, with $I\gamma 2b$ in all conditions but most strongly when $I\gamma 2b$ is normally transcribed in WT, and with $I\gamma 3$, $I\gamma 1$, and $I\gamma 2a$ when they are transcriptionally induced upon stimulation.

To determine if Ikaros binds directly to any of these sequences, we performed EMSA. Exogenous Ikaros, confirmed by supershift, bound strongly to a probe containing a putative Ikaros binding site from HS1,2 (Fig. 8 E). Interestingly, endogenous Ikaros complexes also bound this probe, indicating that Ikaros can directly bind HS1,2 (Fig. 8 F). In contrast, Ikaros did not bind to putative sites from $I\gamma 2b$ or $I\gamma 2a$, and supershift with Ikaros-specific antibodies revealed only low affinity binding to sites in $I\gamma 1$, in keeping with a previous report

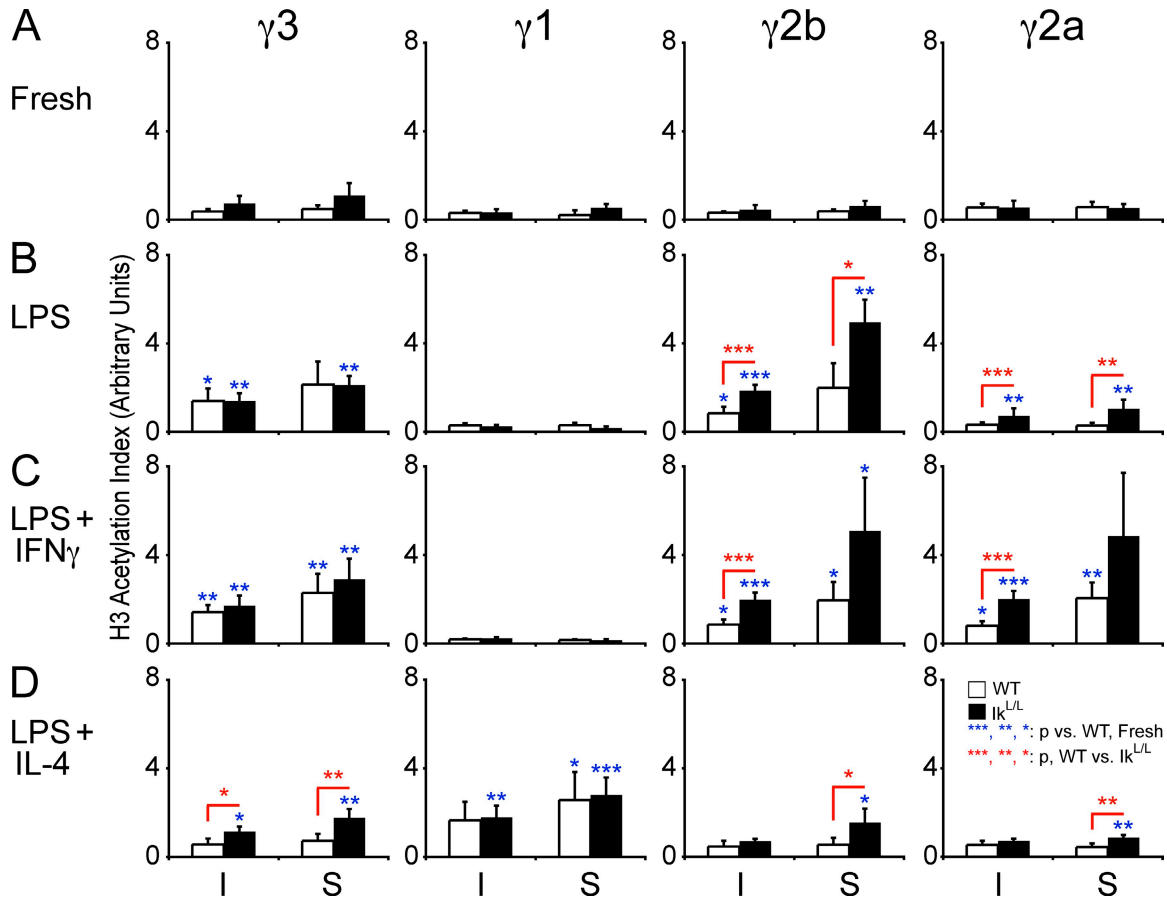


Figure 7. Increased Ach3 at $\gamma 3$, $\gamma 2b$, and $\gamma 2a$ in $Ik^{L/L}$ B cells. WT and $Ik^{L/L}$ $CD43^-$ B cells that were (A) freshly isolated or stimulated for 48 h with (B) LPS, (C) LPS + IFN- γ , or (D) LPS + IL-4 were subjected to ChIP with anti-Ach3 antibodies. Graphs represent the mean I exon (I) and S region (S) Ach3 enrichment indexes plus SD for three independent experiments. Significance was determined by a two-tailed *t* test assuming unequal variance (***, $P < 0.005$; **, $P < 0.02$; *, $P < 0.05$). $S\gamma 2b$ and $S\gamma 2a$ Ach3 levels were consistently higher in $Ik^{L/L}$ versus WT samples after LPS + IFN- γ ($S\gamma 2b$ $Ik^{L/L}/WT$ ratio mean = 2.68 [range = 1.82–3.47]; $S\gamma 2a$ $Ik^{L/L}/WT$ ratio mean = 2.41 [range = 1.45–3.87]).

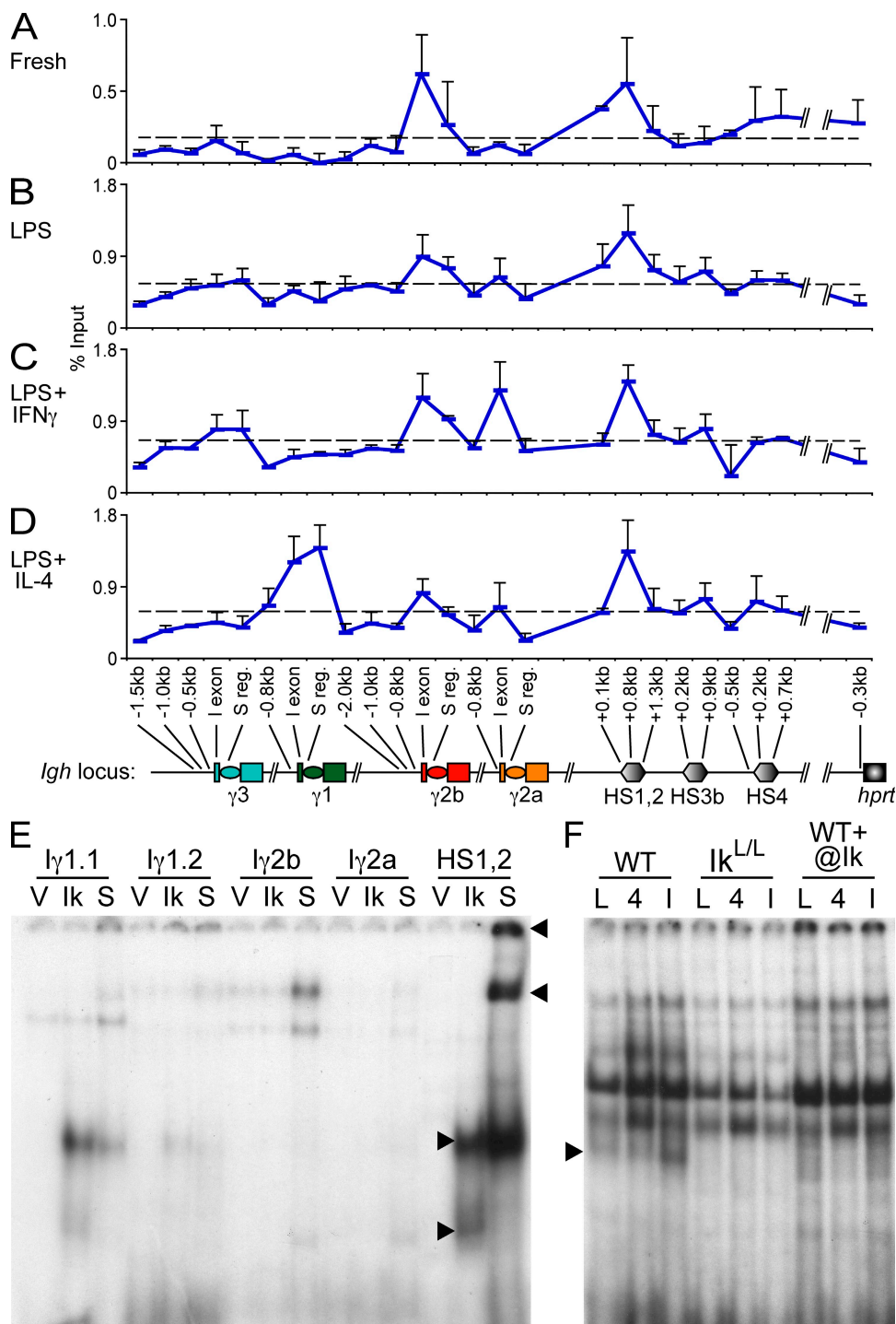


Figure 8. Ikaros associates with C $_H$ promoters and HS1,2 of the 3' enhancer. WT CD43 $^-$ B cells that were (A) freshly isolated or stimulated for 48 h with (B) LPS, (C) LPS + IFN- γ , or (D) LPS + IL-4 were subjected to ChIP-qPCR with anti-Ikaros antibodies. Points represent mean Ikaros enrichment, measured as percentage input, plus SD from two to three independent experiments. Approximate amplicon positions relative to transcription start sites or the 5' ends of HS sites are indicated. Dashed lines represent the mean percentage input for each condition and are defined as the threshold of specific Ikaros binding. (E and F) EMSAs were performed on putative Ikaros binding sites from sequences identified by ChIP (A–D). (E) Nuclear extracts from Cos cells transfected with an empty vector (V) or one encoding the $I\kappa$ 1 isoform (Ik) were incubated with the indicated probes (Table S2). Two putative $I\gamma$ 1 Ikaros binding sites were tested. (F) The HS1,2 probe was incubated with nuclear extracts from WT or $I\kappa^{L/L}$ B cells stimulated for 36 h with LPS (L), LPS + IL-4 (4), or LPS + IFN- γ (I). Specificity was verified by supershift with anti-Ikaros antibodies. Arrowheads indicate Ikaros complexes. Data in E and F are representative of two independent experiments.

(Fig. 8 E) (39). Collectively, these results indicate that Ikaros regulates germline transcription by binding directly to HS1,2, but suggest that its association with I γ regions is indirect, possibly through enhancer–promoter interactions.

Ikaros controls transcriptional competition between S regions

The data described in the previous sections demonstrate that increased and ectopic transcription of γ 2b and γ 2a (and γ 3 with LPS + IL-4) in I $k^{L/L}$ cells correlates with enhanced/ectopic switching to these isotypes. Paradoxically, CSR to IgG₃, IgG₁, and IgE is decreased in I $k^{L/L}$ cells despite normal GLT levels (Figs. 1, 2, and 4). We hypothesized that this discrepancy was caused by increased/ectopic transcription of S γ 2b/S γ 2a (and S γ 3) outcompeting transcription of other S regions for AID-mediated CSR. This model would require that (a) S regions are cotranscribed in individual cells and (b) transcription of S γ 2b/S γ 2a (and S γ 3 with LPS + IL-4) is selectively increased relative to that of other S regions. To test this hypothesis, we examined the expression of *aicda*, and γ 3 and γ 2b GLTs (and *actb* as a positive control) by SC-RT-PCR in LPS- and LPS + IL-4-stimulated (48 h) IgM⁺ cells (Fig. 9 and Table S1). Importantly, because interallelic CSR occurs efficiently in WT cells (40, 41), SC-RT-PCR was performed without regard for allele. In LPS-stimulated WT cells, 97.1% of switching competent cells (e.g., *aicda*⁺) expressed GLTs for γ 2b (90.1%) and/or γ 3 (78.5%), which correlated with switching to these two isotypes (Fig. 1 A; and Fig. 9, B and C). Interestingly, 91.7% of γ 3 GLT⁺ cells also transcribed γ 2b, whereas 15–20% of WT cells switched to IgG₃ after 4 d, indicating that the majority of WT cells cotranscribe S regions and that cells cotranscribing γ 2b and γ 3 can still choose γ 3 for CSR (Fig. 3 and Fig. 9 C). In LPS + IL-4-stimulated cells, the high efficiency of IgG₁ switching suggests that γ 1 GLTs are expressed in almost all cells (Fig. 1 C); γ 1 PCRs were not performed because of the added complexity of performing a fifth PCR in multiplex. Interestingly, 53.9% of *aicda*⁺ WT cells expressed GLTs for γ 3 (34.3%) and/or γ 2b (29.8%), suggesting competition with S γ 1 in a subset of WT cells (Fig. 9, B and C). However, by combining SC-RT-PCR and RT-qPCR data (Fig. 4, B and D), we calculated that the levels of S γ 3 and S γ 2b transcripts per γ 2b⁺/ γ 3⁺ cell were four times lower in LPS + IL-4-stimulated WT cells than in cells stimulated with LPS (Fig. 9 D). These low levels appear insufficient to induce CSR to γ 2b and γ 2a (Fig. 1 C). Thus, S regions are cotranscribed in WT cells and relative S region transcription rates per cell correlate with CSR, indicating that S region competition can occur under normal conditions and may regulate isotype choice.

S region competition is increased in Ikaros-deficient B cells. After LPS stimulation, virtually every *aicda*⁺ I $k^{L/L}$ cell expressed γ 2b GLTs (99.1% γ 2b GLT⁺ cells), whereas the frequency of cells expressing γ 3 GLTs was slightly lower than in WT (64.3% of I $k^{L/L}$ cells, down 14%; Fig. 9 A). Strikingly, every γ 3 GLT⁺ I $k^{L/L}$ cell also expressed γ 2b GLTs, putting S γ 3 in constant competition with S γ 2b (Fig. 9 C). Unlike in WT cells, however, S γ 3/S γ 2b cotranscription in I $k^{L/L}$ cells

was associated with strongly skewed CSR to IgG_{2b} (Fig. 1 A). To explain this difference, we examined per cell transcript levels and found that γ 2b GLTs were expressed at 8.5 times higher levels per γ 2b⁺ cell in I $k^{L/L}$ versus WT cultures, whereas γ 3 GLT levels were similar on a per cell basis (up 1.6-fold in I $k^{L/L}$ vs. WT; Fig. 9 D). Thus, although both WT and I $k^{L/L}$ B cells cotranscribe S γ 3/S γ 2b, increased S γ 2b transcription in I $k^{L/L}$ cells coincides with IgG_{2b}-skewed switching, indicating that higher S γ 2b transcription outcompetes S γ 3 transcription for AID-mediated CSR.

Similarly increased competition was found after LPS + IL-4 stimulation. 91.0% of *aicda*⁺ I $k^{L/L}$ cells expressed γ 3 (57.2%, up 22.9%) and/or γ 2b (83.8%, up 50.8%) GLTs, indicating that these S regions directly compete with S γ 1 for CSR in most cells (Fig. 9, B and C). In addition, LPS + IL-4-stimulated I $k^{L/L}$ cells expressed γ 3 and γ 2b GLTs at per cell levels that were three- to fourfold higher than those found in similarly stimulated WT cells, and nearly equivalent to those observed in LPS-stimulated WT cells (Fig. 9 D). This correlated with ectopic CSR to these isotypes and reduced CSR to IgG₁ (Fig. 1 C). Thus, in LPS + IL-4-stimulated I $k^{L/L}$ cells, high levels of S γ 2b/S γ 3 transcription successfully compete with transcription of S γ 1 for CSR.

Collectively, these observations demonstrate that the majority of B cells cotranscribe S regions, and that per cell transcription rates correlate with isotype selection. Further, our results strongly support the hypothesis that increased transcription of S γ 2b and S γ 2a (and S γ 3 with LPS + IL-4) in I $k^{L/L}$ cells outcompetes transcription of other S regions for CSR and thus skews isotype selection.

DISCUSSION

In this study, we identify Ikaros as a critical regulator of Ig isotype selection in B cells during CSR. Ikaros deficiency results in increased and ectopic switching to IgG_{2b} and IgG_{2a} (and ectopic switching to IgG₃ with LPS + IL-4) and enhanced overall switching, but reduced switching to IgG₃ (with LPS and LPS + IFN- γ), IgG₁, IgE, and IgA. This function is independent of Ikaros's role in B cell development, as inhibition of Ikaros in mature WT cells skews isotype selection to IgG_{2b} and IgG_{2a}, whereas retroviral expression of Ikaros in I $k^{L/L}$ B cells rescues CSR. Further, in direct correlation with their CSR phenotype, I $k^{L/L}$ cells exhibit sharp increases in transcription and AcH4, an AID-dependent histone mark, at S γ 2b and S γ 2a (and S γ 3 with LPS + IL-4) but not other S regions. Ikaros directly binds the *Igh* locus in vivo and is required to prevent hyperacetylation of histone H3 at the γ 2b and γ 2a (and γ 3 with LPS + IL-4) genes upon B cell activation. Thus, Ikaros maintains repressive chromatin at γ 2b and γ 2a (and γ 3 with LPS + IL-4), and suppresses transcription, AID accessibility, and switching to these isotypes. These results identify Ikaros as the first factor that controls the range of isotypes targeted by CSR.

Our results also reveal that the choice to undergo CSR to specific C_H genes is controlled by the balance of germline transcription across different S regions within individual cells.

We have shown that the majority of *aicda*⁺ LPS-stimulated WT cells coexpress GLTs for $\gamma 2b$ and $\gamma 3$, suggesting competition between these two isotypes for switching. Further, selective increases in $\gamma 2b$ GLTs in *Ik*^{L/L} cells, where $\gamma 3$ is always co-transcribed with $\gamma 2b$, correlate with increased CSR to IgG_{2b} and decreased CSR to IgG₃. Thus, repression of S $\gamma 2b$ tran-

scription by Ikaros is required to maintain a balance between S $\gamma 2b$ and S $\gamma 3$ transcription, allowing for normal switching to both isotypes. Similarly, Ikaros fine tunes S $\gamma 2b$ and S $\gamma 2a$ transcription in response to LPS + IFN- γ , to allow switching to IgG₃. Therefore, in addition to suppressing promiscuous switching to IgG_{3/2b/2a} in response to LPS + IL-4, and

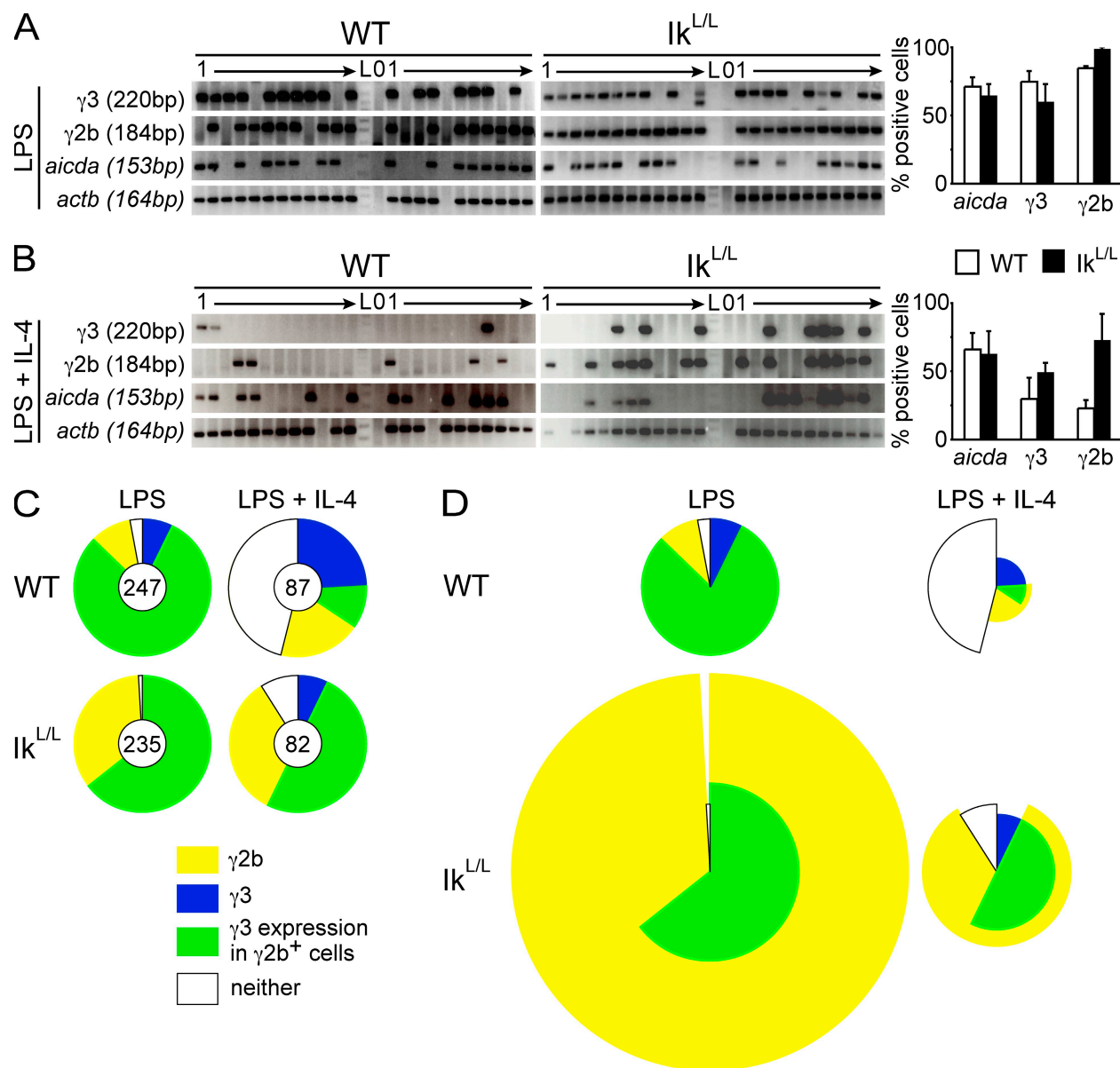


Figure 9. Increased S region competition at the SC level for CSR in *Ik*^{L/L} B cells. SC-RT-PCR was performed on IgM⁺ WT and *Ik*^{L/L} cells after 48 h of stimulation and two divisions (assessed by CFSE dilution). (A and B) Representative data from cohorts of (A) LPS- and (B) LPS + IL-4-stimulated cells are shown. Only *actb*⁺ wells were counted (positive control for sorting). Bar graphs represent mean percentages plus SD of cells positive for *Aicda*, or $\gamma 3$ or $\gamma 2b$ GLTs, from two independent experiments (0, no cells; 1, one cell; raw data are shown in Table S1). (C) Pie charts represent mean percentages of *aicda*⁺ cells expressing GLTs for $\gamma 3$, $\gamma 2b$, both, or neither. The numbers of *aicda*⁺ cells analyzed over two experiments are indicated (chart centers). (D) Data from C was integrated with GLT expression levels measured by RT-qPCR (Fig. 4, B and D) to give a comprehensive view of $\gamma 2b$ and $\gamma 3$ GLT expression in individual cells. Pie chart wedges represent the percentages of *aicda*⁺ B cells that express the indicated GLTs. Wedge radii are defined such that the wedge area corresponds to total GLT expression levels; $\gamma 3$ and $\gamma 2b$ GLT levels were normalized to LPS-stimulated WT B cells, for which the radius was set to 1. Thus, the percentage of degrees out of 360 taken by each wedge corresponds to the percentage of expressing cells, and the wedge area corresponds to the integrated per cell expression level.

IgG_{2a} in response to LPS, Ikaros limits transcription across S γ 2b and S γ 2a so that germline transcription of other S regions (i.e., S γ 3 and S α) can effectively compete for switching. These results indicate that the isotype fate of individual cells during CSR is determined by the relative levels of germline transcription across different S regions. Considering that single human B cells have also been reported to transcribe multiple S regions simultaneously (42), this is likely a general mechanism, across species, for isotype selection during CSR.

A question arising from our work is how Ikaros suppresses transcription at γ 3, γ 2b, and γ 2a. Interestingly, we have found that histone H3 is hyperacetylated at these genes in Ik^{L/L} cells. Although it is possible that these increased AcH3 levels result from transcription-coupled processes, it is attractive to speculate that Ikaros regulates germline transcription by maintaining a repressive chromatin structure. In this respect, it is well documented that histone acetylation contributes to transcription by opening chromatin and/or providing binding platforms for regulatory factors (43, 44).

Considering that H3 is hyperacetylated in Ik^{L/L} cells and that Ikaros interacts with the Sin3 and NuRD histone deacetylase (HDAC) complexes (45–48), we expected Ikaros to recruit these complexes to the *Igh* locus to regulate germline transcription. However, we have been unable to find reduced *Igh* locus occupancy for Sin3a, Mi-2 β (a core member of NuRD), HDAC1, or HDAC2 in Ik^{L/L} versus WT cells by ChIP-qPCR or double cross-linking (DC)-ChIP-qPCR (Fig. S10). Furthermore, shRNA knockdown of Mi-2 β in WT B cells neither increased γ 2b transcription nor altered switching specificity (Fig. S11). These data indicate that Ikaros does not suppress H3 acetylation and germline transcription by recruiting the NuRD and Sin3 HDAC complexes to the *Igh* locus.

Our results indicate that Ikaros may directly regulate germline transcription through the HS1,2 region of the *Igh* 3' enhancer. Ikaros associates with HS1,2 in all conditions tested, and Ikaros proteins bind directly to a sequence in this region in vitro. In contrast, despite the association of Ikaros with the I γ 1, I γ 2b, and I γ 2a regions by ChIP, we were unable to identify sequences in these regions that could be strongly and directly bound by Ikaros. Although we cannot rule out binding to noncanonical sites, these data suggest that Ikaros binds directly to HS1,2 and interacts indirectly with I γ 1, I γ 2b, and I γ 2a. Our 3C data, as well as those from Wuerffel et al. (16), support this interpretation, as HS1,2 interacts with fragments containing the γ 3, γ 1, γ 2b, and γ 2a I promoters/exons. Interestingly, HS1,2- γ 1 interactions increase when this gene is transcribed (e.g., LPS + IL-4; Figs. 4 and 6) (16), which correlates with the appearance of an Ikaros binding peak by ChIP. Similarly, Ikaros associates with I γ 2a only when cells are stimulated with LPS + IFN- γ , which induces both γ 2a GLTs and increased HS1,2-I γ 2a interactions. Thus, we propose that Ikaros interacts indirectly with and regulates C_H gene promoters through HS1,2.

The idea that Ikaros mediates repression through the HS1,2 regulatory region of the 3' enhancer is bolstered by the observation that 3' enhancer disruption most drastically

affects transcription of and CSR to γ 3, γ 2b, and γ 2a, the C_H genes most strongly affected by Ikaros deficiency (14, 15). One issue with this model is that HS1,2 deletion does not affect CSR (14), suggesting that HS1,2 may be not be functionally important for CSR and/or that it functions independently of Ikaros. This explanation, however, is probably too simplistic. In another complex locus, the β -globin locus, deletions of individual regulatory elements result in little to no phenotype, whereas combined deletions can drastically inhibit globin gene expression, indicating that the loss of individual elements can be compensated for by redundant functions in other regions (49). Thus, compensation by other 3' enhancer elements, which have many similar transcription factor motifs (50), could explain the lack of CSR phenotype in HS1,2^{-/-} cells. In addition, HS1,2 can strongly synergize with other HS fragments to activate transcription (51), and the core region HS1,2 is highly conserved between mouse, rat, rabbit, and human (52), indicating that HS1,2 is likely to play important roles in CSR.

How then, can HS1,2^{-/-} and Ik^{L/L} B cell CSR phenotypes be reconciled? An obvious possibility is that Ikaros could repress germline transcription and histone acetylation by antagonizing positive regulatory functions at this element. In this case, HS1,2 deletion would abolish both negative regulation by Ikaros and positive regulation by unknown factors, resulting in a neutral phenotype. In contrast, Ikaros deficiency would allow increased activity by positively acting factors, resulting in a significant phenotype. Interestingly, Ikaros has been shown to compete with positive regulators to modulate the transcription of target genes in other settings (22, 53, 54). In this respect, our EMSA assay indicates that multiple factors can bind at or near the HS1,2-Ikaros binding site (Fig. 8 F). Thus, it will be important to determine if Ikaros competes with other factors for binding to the HS1,2 regulatory element to fine tune germline transcription.

Finally, the efficiency of CSR is controlled by several mechanisms, including cell proliferation, AID expression, subcellular localization and posttranslational modifications, DSB formation, DNA repair, S region synapsis, and end joining (1). Our results suggest that S region transcription rates in WT cells also limit switching efficiency. This concept is supported by (a) the low rates of CSR compared with switching competency in WT cells (~30% of cells switched after 4 d with LPS, whereas 70% were *aicda*⁺GLT⁺ after 48 h; Fig. 3, Fig. 9A, and Table S1), (b) the similar levels of CSR-competent cells in Ik^{L/L} and WT cultures (67% of Ik^{L/L} cells were *aicda*⁺GLT⁺ after 48 h; Fig. 9A and Table S1), and (c) the direct correlation between increased per cell GLT levels, AID accessibility, and switching frequency in Ik^{L/L} cells (~50% switched after 4 d; Fig. 5 B, Fig. 9 D, and Fig. S5 B). Why it would be advantageous to limit S region transcription rates and CSR efficiency under normal conditions is not clear. This mechanism could result in more flexible humoral immune responses in vivo, as slowing the rate of CSR may prevent B cells from switching en masse soon after activation, thus allowing isotype selection to be modified over time. In addition, reduced S region transcription, and therefore DSB formation, would decrease the probability of

unrepaired DSBs, which can lead to oncogenic translocations (55).

In summary, our results reveal transcriptional competition between constant region genes as a central and general mechanism for isotype specification during CSR. We have shown that Ikaros is a master regulator of this competition.

MATERIALS AND METHODS

Mice. The $I\kappa B^{\Delta L}$ mouse line was previously described (21). Mice backcrossed >10 times onto the C57BL/6 genetic background were analyzed at 5–9 wk of age. All animal work was performed under protocols approved by the Direction des Services Vétérinaires du Bas-Rhin, France (authorization no. 67-343).

Cell culture. Spleen cells were used for all experiments. B cells (B220⁺, IgM⁺CD43⁻, or CD43⁻IgG/A⁻ cells; >98% purity) were sorted on a FACSVantage SE option DIVA (BD) or enriched by depletion of CD43⁺ cells with MACS beads (>90% purity), and CD4⁺ T cells were isolated with anti-CD4 MACS beads (>90% purity; Miltenyi Biotech). Cells were labeled with 5 µg/ml CFSE (10 min at 37°C; Sigma-Aldrich) and were maintained at 1.2×10^6 cells/ml in complete medium (RPMI 1640, 10% FCS, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, and 1% antibiotics) with 25 µg/ml LPS (serotype 0111:B4 from *Escherichia coli*; Sigma-Aldrich), 5 ng/ml IL-4 (Sigma-Aldrich), 100 ng/ml IFN-γ (PeproTech), 3 ng/ml TGF-β (R&D Systems), 5 ng/ml IL-5 (BD), and 0.5 ng/ml PMA (Sigma-Aldrich).

Flow cytometry. Reagents included anti-B220-FITC (RA3-6B2), anti-mouse IgG_{2b}-biotin (RMG2b-1; BioLegend), anti-mouse IgA-PE (Southern-Biotech), F(ab')₂ goat anti-mouse IgM-Cy5 and streptavidin-Cy5 or -PE (Jackson ImmunoResearch Laboratories), anti-mouse IgG₃-biotin (R40-82), anti-mouse IgG₁-biotin (A85-1), anti-mouse IgG_{2a}^b-biotin (Igh-b; 5.7) and anti-CD43-PE (BD), goat anti-mouse IgG (H+L)-biotin (Invitrogen), anti-NGFR-Cy5 (8737; provided by W. Pear, University of Pennsylvania, Philadelphia, PA), and 7-aminoactinomycin D (Sigma-Aldrich). Cells were analyzed with a FACSCalibur (BD) and FlowJo software (Tree Star, Inc.).

RT-qPCR. RNA was isolated with RNeasy kits (QIAGEN). 350 ng RNA was reverse transcribed in 20 µl (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 500 µM dNTP, 10 U recombinant RNasin ribonuclease inhibitor [Promega], 0.5 µM oligo d(T) [New England Biolabs, Inc.], and 40 U SuperScript II RT [Invitrogen]). SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) was used for all qPCRs. Approximately 3 ng of cDNA was run (in triplicate) on a LightCycler 480 (96-well plate format) and analyzed with LightCycler 480 basic software (Roche). Transcript quantities for each gene were calculated relative to standard curves and normalized to *Igβ* transcripts. Gene of interest/*Igβ* ratios for each condition were averaged across experiments and normalized to the indicated WT condition, to give relative expression versus WT or versus unstimulated WT, as noted. Table S2 lists oligonucleotides for all experiments.

SC-RT-PCR. Two rounds of PCR were performed with fully nested primers, as previously described (54). Only *actb*⁺ wells were analyzed (>90%).

Retrovirus production and transduction. MigR1-NGFR (provided by W. Pear) is a murine stem cell virus-based retrovirus (56). Ik1 cDNA was amplified from mouse spleen cDNA by PCR (bp 271–1,818; available from GenBank/EMBL/DBJ under accession no. NM_001025597). Ik1* cDNA was obtained by site-directed mutagenesis to delete exon 2–derived Ik1 sequences (bp 310–430). Ik6 cDNA was obtained by fusing PCR fragments for exons 1 and 2 (bp 271–429) and exon 7 (bp 1,117–1,818) of Ik1. cDNAs were cloned into the MigR1-NGFR vector and were designated Ik1-NGFR, Ik1*-NGFR, and Ik6-NGFR. pQsupR-Mi2 (Mi-2β shRNA; provided by S. Smale, University of California, Los Angeles, Los Angeles, CA) and pQsupR

(mock) vectors were previously described (57). Vectors were transfected into Eco-Phoenix packaging cells (provided by G. Nolan, Stanford University, Stanford, CA) to produce high-titer retroviral supernatants. 3.5×10^5 CFSE-stained cells (1.2×10^6 cells/ml) were cultured in LPS for 24 h, transduced with retroviral supernatant (25% total vol; 4 µg/ml polybrene; 25 µg/ml LPS), and centrifuged for 90 min at 2,600 rpm. Medium was replaced after 12 h and cells were analyzed 60 h later.

ChIP. The ChIP protocol was adapted from Wang et al. (18) and Millipore (<http://www.millipore.com/techpublications/tech1/mcproto407>). In brief, 1.8×10^7 B cells were cross-linked at 37°C for 10 min in 5 ml PBS/0.5% BSA/1% ultra-pure formaldehyde (Electron Microscopy Sciences). After quenching with 0.125 M glycine and a cold PBS wash (with 1× protease inhibitor cocktail [PIC]; Roche), cells were lysed in 5 ml of Triton X lysis buffer for 10 min on ice (1% Triton X-100, 50 mM MgCl₂, 100 mM Tris-HCl [pH 7.1], 11% sucrose, 1× PIC). Nuclei were pelleted at 2,000 rpm for 10 min (4°C) and were lysed in 500 µl of SDS lysis buffer for 10 min on ice (1% SDS, 50 mM Tris-HCl, 10 mM EDTA, 1× PIC). Chromatin was sonicated to 500–1,000 bp using a Bioruptor 200 (Diagenode), and sonication efficiency was checked. After 2× dilution in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl), chromatin was precleared by rotating for 2 h at 4°C with 80 µl 50% protein A slurry (0.2 mg/ml sheared salmon sperm DNA, 0.5 mg/ml BSA, 50% protein A; GE Healthcare). 1.3×10^6 cell equivalents were saved as input. 3.9×10^6 cell equivalents were incubated overnight with 2 µg anti-AcH3 (Millipore), 2 µg anti-AcH4 (Millipore), 2.5 µg anti-Ikaros (rabbit anti-mouse produced in house), anti-Mi-2β (5 µl of rabbit serum; gifts from P. Wade [National Institute of Environmental Health Sciences, Research Triangle Park, NC] and S. Smale), 5 µg anti-Sin3a (Santa Cruz Biotechnology, Inc.), 5 µg anti-HDAC1 (Abcam), 5 µg anti-HDAC2 (Abcam), and control antibodies (Bethyl Laboratories). ChIPs were recovered with 65 µl 50% protein A slurry for 5 h and processed according to the Millipore protocol. ChIP and input DNA was dissolved in 140 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), and inputs were diluted 10 times. 2-µl aliquots were analyzed (in duplicate) by qPCR. Calculations were as follows: percentage input (%I) = $100 \times ([\text{antibody bound}] - [\text{IgG bound}]) / (\text{input} \times 10)$; and histone acetylation index = $(\%I^{\text{Igh sequence}}) / (\%I^{\text{Hprt promoter}})$. For DC-ChIP, cells were first fixed with disuccinimidyl glutarate (58).

3C. 3C was performed as previously described (59) with some modifications. In brief, 11×10^6 cells were fixed for 10 min in 10 ml 1× PBS/0.5% BSA/1.5% formaldehyde (Electron Microscopy Sciences) at room temperature with tumbling (10 rpm). Fixation was quenched with 0.125 M glycine. Cells were washed one time in cold PBS/1× PIC and lysed for 10 min on ice in 1 ml of lysis buffer (10 mM Tris [pH 8], 10 mM NaCl, 0.2% NP-40, 1× PIC). After centrifugation, cells were resuspended in 537 µl of digestion buffer (60 µl of 10× buffer R [Fermentas], 1× PIC). SDS was added to 0.3% and samples were rotated for 1 h at 37°C (99 rpm). Triton X was added to 1.8% to sequester SDS, bringing the total volume to 600 µl. Samples were rotated (99 rpm) for 1 h at 37°C before overnight digestion with 500 U HindIII (50 rpm; New England Biolabs, Inc.). HindIII was inactivated by adding SDS to 1.75% and incubating for 20 min at 65°C. 10^6 cell equivalents were removed, de-cross-linked, and analyzed by qPCR to monitor digestion efficiency (routinely 80–90%; Fig. S9 A). 10^7 cell equivalents were diluted to 8 ml in 1× ligation buffer (New England Biolabs), 1% Triton X, and 1× PIC, and SDS was sequestered by rocking for 1 h at 37°C. 4,000 NEB U of ligase (New England Biolabs, Inc.) were added and samples were rocked for 10 min at 4°C before incubation for 6 h at 16°C, followed by 30 min at room temperature. Samples were de-cross-linked overnight at 65°C with 300 µg protease K and were retreated with 300 µg protease K for 1 h at 45°C. DNA was extracted using ultra-pure phenol/chloroform/isoamyl alcohol (25:24:1; Invitrogen), washed two times with chloroform, ethanol precipitated with 2.5 M ammonium acetate, and washed four times with 75% ethanol. After suspension in TE, relative DNA concentrations were calculated by qPCR (ChIP primers “HS4 –0.5 kb”).

Ligation products were analyzed by qPCR in duplicate or triplicate using ~200 ng DNA. Absolute quantities were calculated using a template consisting of equimolar ratios of all possible ligation products. The template was constructed as previously described (60) with PCR fragments from the *Igh* and *Gapd* loci and diluted in genomic DNA. qPCR specificity was confirmed with negative controls (without fixation, digestion, or ligation), melting point analysis, digestion of PCR products with HindIII, and migration of PCR and digestion products on 2% agarose gels (Fig. S9 B). Relative cross-linking frequency was calculated as follows: cross-linking frequency = $([HS1,2 - \text{fragment X}]/[HS4 - 0.5 \text{ kb loading control}])/([Gapd 3' - Gapd 5']/[HS4 - 0.5 \text{ kb}])$.

EMSA. Nuclear extracts (NEs) and EMSAs were prepared as previously described (61). 2.5 μ g NE from Cos cells transfected with mock cDNA or cDNA coding for I κ 1 proteins or 4 μ g NE from CSR-induced B cells was used.

Online supplemental material. Table S1 summarizes raw data from SC-RT-PCR experiments. Table S2 lists oligonucleotides. Fig. S1 shows statistical analysis of WT and I κ ^{L/L} CSR frequencies after LPS, LPS + IL-4, LPS + IFN- γ , and LPS + IL-5 + TGF- β stimulation. Fig. S2 quantifies Ig isotype production by WT and I κ ^{L/L} B cells using ELISA. Fig. S3 analyzes WT and I κ ^{L/L} CSR frequency as a function of proliferation. Fig. S4 examines CSR in whole and bona fide unswitched WT and I κ ^{L/L} B cells. Fig. S5 shows the effect of retroviral expression of dominant-negative Ikaros on CSR in WT cells, and full-length and exon 2–deleted Ikaros isoforms on CSR in I κ ^{L/L} B cells. Fig. S6 shows RT-qPCR analysis of *aicda* expression in stimulated WT and I κ ^{L/L} cells. Fig. S7 shows SC-RT-PCR analysis of *aicda*, γ 3 GLT, and γ 2b GLT expression in freshly isolated IgM⁺CD43⁻ B cells and CD4⁺CD3⁺ T cells. Fig. S8 shows RT-qPCR analysis of μ , γ 3, γ 2b, and γ 2a GLT expression in WT and I κ ^{L/L} B cells after 48 h with LPS and one or three divisions. Fig. S9 shows digestion efficiency and PCR specificity controls for 3C experiments. Fig. S10 shows ChIP-qPCR and DC-ChIP-qPCR for Mi-2 β , Sin3a, HDAC1, and HDAC2 at the *Igh* locus in WT and I κ ^{L/L} cells. Fig. S11 shows the effect of Mi-2 β shRNA knockdown on CSR in WT B cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082311/DC1>.

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