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RESEARCH COMMUNICATION

Flexible ordering of antibody class switch and V(D)J joining during B-cell ontogeny

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V(D)J joining is mediated by RAG recombinase during early B-lymphocyte development in the bone marrow (BM). Activation-induced deaminase initiates isotype switching in mature B cells of secondary lymphoid structures. Previous studies questioned the strict ontological partitioning of these processes. We show that pro-B cells undergo robust switching to a subset of immunoglobulin H (IgH) isotypes. Chromatin studies reveal that in pro-B cells, the spatial organization of the *Igh* locus may restrict switching to this subset of isotypes. We demonstrate that in the BM, V(D)J joining and switching are interchangeably inducible, providing an explanation for the hyper-IgE phenotype of Omenn syndrome.

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Humoral immunity is dependent on antigen receptors that are assembled from immunoglobulin (Ig) heavy chain V_H, D, and J_H segments during early B-cell development. RAG recombinase mediates V(D)J gene segment assembly in the bone marrow (BM) (Zhang et al. 2010; Schatz and Ji 2011). In mature B cells located in secondary lymphoid structures, activation-induced deaminase (AID) initiates a switch from IgM expression to secondary Ig classes (IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) that have specialized effector functions (Stavnezer 1996, 2000). Class switch recombination (CSR) occurs via an intrachromosomal deletion process while maintaining the original V(D)J assembly (Kenter 2012). The failure to assemble V(D)J exons or signal through the B-cell receptor (BCR) creates a block in the developmental progression of pro-B cells (Jankovic et al. 2004; von Boehmer and Melchers 2010). However, the strict ontological separation of V(D)J

joining and CSR may break down under some circumstances (Milili et al. 1991; Rolink et al. 1996; Weller et al. 2001; Dudley et al. 2002; Mao et al. 2004; Han et al. 2007; Ueda et al. 2007; Scheeren et al. 2008; Kuraoka et al. 2009; Wesemann et al. 2011). For example, in μ MT mice, membrane Ig μ is not expressed, and B-cell development is halted at the pro-B-cell stage; nevertheless, IgA is selectively expressed (Melamed et al. 2000; Macpherson et al. 2001), implying an alternative pathway for CSR that can circumvent the pro-B-cell block. However, it is unclear where and under what circumstances the switch to IgA occurs.

Omenn syndrome is a severe combined immunodeficiency disease associated with autoimmunity and atopy, most commonly arising from hypomorphic mutations in the RAG recombinase genes. The syndrome is characterized by the severe paucity of B and T lymphocytes, hypog γ -globulinemia, and, paradoxically, elevated IgE levels (Wong and Roth 2007; Ozcan et al. 2008). To account for hyper-IgE in Omenn syndrome, we hypothesized that (1) CSR occurs in BM pro-B cells prior to V(D)J joining, (2) CSR is predisposed to IgE, and (3) V(D)J joining events can follow CSR. Here, we show that *Rag*-deficient or *Mb1*-deficient pro-B cells can be induced to undergo robust CSR, albeit to a restricted subset of Ig constant (C_H) region genes, including IgG2b and IgE, prior to or following V(D)J joining. Immunization of *Rag1*^{-/-} mice with LPS promotes IgG2b switching in BM pro-B cells. We also demonstrate that V(D)J joining can follow CSR in Abelson transformed pro-B-cell and pre-B-cell lines. Studies of *Igh* locus chromatin structure revealed a unique stage-specific organization in pro-B cells that is correlated with preferential IgG2b and IgE switching. The flexible expression of the V(D)J recombination and CSR programs has implications for the genesis of Omenn syndrome hyper-IgE, autoimmune repertoire development, and leukemagenesis (Tsai et al. 2008).

Results and Discussion

To test the proposition that robust CSR can occur in BM pro-B cells prior to V(D)J recombination, we evaluated *Rag2*, *AID*, and isotype-specific germline transcript (GLT) expression in an *E2A*^{-/-} pre-pro-B cell line, in pro-B cells isolated from *Rag1*- or *Mb1*-deficient mice, and in a panel of Abelson transformed pro-B-cell lines (*Rag*-deficient R2K2 and 445.3) and pre-B-cell lines (PA112.1, PA112.2, PA48.1, A70.2, and ATM2A). *E2A* and *Rag* deficiencies preclude V(D)J joining, whereas *Mb1*-deficient pro-B cells assemble V(D)J exons but cannot signal through the pre-BCR and are blocked at the pro-B-cell stage (Pelanda et al. 2002). Elevated *Rag2* but not *AID* transcripts are detected in all strains of unstimulated pre-pro-B and pro-B cells but not in the Abelson transformed cell lines, as expected (Fig. 1A, top; Supplemental Fig. S1; Muljo and Schlessel 2003). Upon activation with the CSR inducers LPS+CD40L

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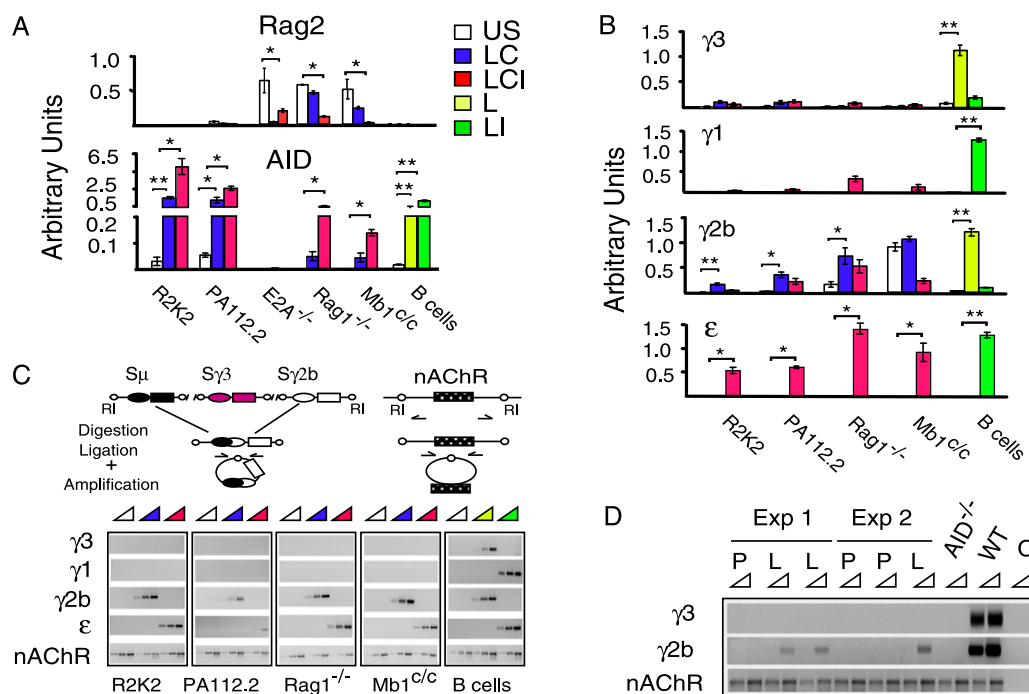


Figure 1. Pro-B cells undergo inducible CSR. *E2A*-deficient pre-pro-B cells, *Rag1*-deficient or *Mb1*-deficient pro-B cells, Abelson transformed pro-B-cell lines (R2K2), and pre-B-cell lines (PA112.2) were unstimulated (US) or activated with LPS+CD40L (LC) or LPS+CD40L+IL4 (LCI), and wild-type (WT) resting splenic B cells were unstimulated or activated with LPS (L) or LPS+IL4 (LI). (*) $P \leq 0.05$; (**) $P \leq 0.001-0.0001$. (A,B) Quantitative RT-PCR assays for Rag2 and AID (A) and GLTs $\gamma 3$, $\gamma 1$, $\gamma 2b$, and ϵ (B) from five samples from three to five independent experiments were normalized to the rRNA 18S transcript. (C, top) DC-PCR assay schematic, EcoRI (RI) sites, and *nAChR* gene loading control. (Bottom) DC-PCR analyses of $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \gamma 2b$, and $\mu \rightarrow \epsilon$ CSR are representative of three independent experiments. (D) DC-PCR analysis of pro-B cells from *Rag1*^{-/-} mice injected with LPS or PBS. Wild-type or *AID*^{-/-} splenic B cells activated with LPS or no template control (C) are representative of three independent experiments.

or LPS+CD40L+IL4, *Rag2* expression is reduced in *E2A*^{-/-} pre-pro-B cells and *Rag1*-deficient and *Mb1*-deficient pro-B cells (Fig. 1A). In contrast, AID expression is markedly induced in *Rag1*-deficient and *Mb1*-deficient pro-B cells but not *E2A*^{-/-} pre-pro-B cells (Fig. 1A, bottom). Similarly, in R2K2 and PA112.2 as well as other Abelson transformed cell lines, AID expression substantially increases in response to CSR stimuli (Fig. 1A, bottom; Supplemental Fig. S1A,B). *E2A*^{-/-} pre-pro-B cells were not further considered because AID is not expressed. Previous studies of BM pro-B cells concluded that AID transcription was undetectable (Crouch et al. 2007) but did not evaluate expression in response to CSR activators.

We next examined the pattern of GLT expression in pro-B cells. C_H genes are organized in transcription units that include a noncoding intronic (I) exon, the switch (S) region, and the C_H exons. Downstream S regions are targeted for recombination with S_μ by selective activation of GLT promoters in response to specific stimuli. GLTs initiate from a promoter located upstream of each I exon and terminate 3' of the C_H region (Stavnezer 2000). Typically, GLTs $\gamma 3$ and $\gamma 2b$ are induced by LPS or LPS+CD40L, whereas GLTs $\gamma 1$ and ϵ also require IL4 in mature B cells (Fig. 1B). However, in response to the appropriate CSR stimuli, *Rag1*-deficient and *Mb1*-deficient pro-B cells and the Abelson transformed cell lines expressed GLT $\gamma 2b$ and ϵ but not $\gamma 1$ and $\gamma 3$ (Fig. 1B; Supplemental Fig. S1A). Accordingly, expression of GLT ϵ and not $\gamma 1$ was noted for immature B cells (Wesemann et al. 2011).

Activation of AID expression and germline transcription prompted us to investigate whether CSR occurs in pro-B or pre-B cells. We used semiquantitative digestion circularization PCR (DC-PCR) to detect CSR events, since pro-B cells do not express surface Ig (Fig. 1C, top). The nonrearranging *Acetylcholine receptor* (*nAChR*) gene was used as a loading control (Fig. 1C). DC-PCR products representing $\mu \rightarrow \gamma 2b$ and $\mu \rightarrow \epsilon$ CSR events are detected in the *Rag1*-deficient and *Mb1*-deficient pro-B cells and Abelson transformed cell lines, whereas $\mu \rightarrow \gamma 3$ or $\mu \rightarrow \gamma 1$ events are not detected in either case (Fig. 1C, bottom; Supplemental Fig. S2). In contrast, in mature splenic B cells activated with LPS or LPS+IL4, a full spectrum of $\mu \rightarrow \gamma 3/\gamma 2b$ and $\mu \rightarrow \gamma 1/\epsilon$ CSR events is detected, respectively (Fig. 1C). This restricted pattern of CSR in the pro-B versus mature B cells parallels their GLT expression profiles. Strikingly, the levels of DC-PCR products in primary pro-B cells and activated splenic B cells were similar, indicating robust CSR in early B cells (Fig. 1C; Supplemental Fig. S2).

High-frequency $\mu \rightarrow \gamma 2b/\epsilon$ CSR in pro-B cells was confirmed by several independent methods, including analysis of post-switch transcripts (PSTs), circle transcript PCR (CT-PCR) assays, and S/S junction analyses. Following CSR, the I_μ exon is in close proximity with a new downstream C_H region, thereby permitting PST expression that was detected using a forward primer in I_μ together with a reverse primer in the relevant C_H exon. PST $\gamma 2b$ and ϵ are induced in *Rag1*-deficient and *Mb1*-deficient pro-B cells and Abelson transformed cell lines

upon activation with CSR stimuli (Supplemental Fig. S3). CT-PCR assays indicate that $\mu \rightarrow \gamma 2b$ or $\mu \rightarrow \varepsilon$ CSR occurs dynamically in *Rag1*^{-/-} pro-B cells and Abelson transformed cell lines (Supplemental Fig. S4). We sequenced S/S recombination junctions from appropriately activated *Rag1*^{-/-} pro-B cells or R2K2 cells and found that S μ joined to a variety of sites in S $\gamma 2b$ or S ε (Supplemental Figs. S5–S8). Switch junctions were blunt or contained short junctional microhomologies or occasional insertions in normal proportions (Stavnezer et al. 2010) and confirmed that the mechanism of CSR in pro-B cells is operationally indistinguishable from that observed in mature B cells.

To determine whether CSR can be induced in vivo, *Rag1*^{-/-} mice were injected with LPS or PBS, and pro-B cells were isolated directly from the BM and tested for CSR by DC-PCR. Wild-type or *AID*-deficient splenic B cells that were activated with LPS in ex vivo culture served as positive and negative controls for CSR, respectively. *nAChR* amplification is shown as a loading control, and the absence of PCR products in reactions devoid of template demonstrate PCR specificity. Under these activation conditions, $\mu \rightarrow \varepsilon$ CSR is negligible. Switching $\mu \rightarrow \gamma 2b$ but not $\mu \rightarrow \gamma 3$ was observed in 67% (six out of nine) of LPS immunized mice ($P = 0.007$; χ^2) but not (zero out of six) for PBS-injected controls from three independent experiments (Fig. 1D). Targeting of CSR to the $\gamma 2b$ but not the $\gamma 3$ locus reflects the established GLT expression pattern that we observed for pro-B cells activated in ex vivo culture (Fig. 1B,C). We conclude that CSR can occur in BM *Rag1*^{-/-} pro-B cells prior to V(D)J recombination.

To address the question of whether a B cell that experienced CSR can subsequently undergo V(D)J joining, switched clones from the R2K2 pro-B-cell line were isolated by limiting dilution. CSR was confirmed in clones B5 ($\mu \rightarrow \gamma 2b$) and A2 ($\mu \rightarrow \varepsilon$) by DC-PCR, and clonality was demonstrated by virtue of unique intra-S μ region rearrangements (Fig. 2A, Supplemental Fig. S9). Treatment of these cell lines with STI571, a pharmacologic inhibitor of Abl kinase activity, resulted in activation of V(D)J joining and differentiation to the late pre-B-cell state (Muljo and Schlissel 2003). STI571 stimulation of B5 and A2 clones led to induction of the V(D)J joining gene program (Fig. 2B); Muljo and Schlissel 2003). The *Rag2*⁺ but not the empty expression construct induced diverse D_{DFL16.1}-J_{H1} coding joins (CJs), while PST expression persisted (Fig. 2C; Supplemental Figs. S10, S11). Thus, pro-B-cell clones that experienced CSR retain the ability to undergo V(D)J recombination. Importantly, evidence indicates that BM pro-B cells expressing IgG1 ($\gamma 2b$ chains (Storb et al. 1994) are capable of mature B-cell development that is indistinguishable from wild type.

To determine whether class-switched pre-B cells could complete the developmental program of recombination, we assayed V–J joining at the light chain *Ig κ* locus.

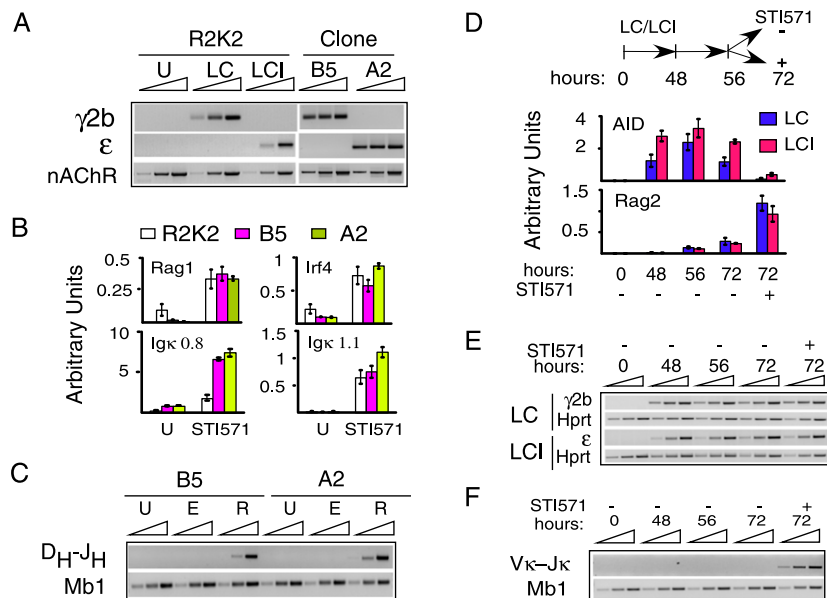


Figure 2. V(D)J joining can follow CSR in early B-cell lines. Cells were untreated (U) or activated with LPS+CD40L (LC) or LPS+CD40L+IL4 (LCI). Results are representative of three to nine samples from three independent experiments. (A) DC-PCR analysis of CSR in R2K2 and in clones A2 and B5. (B) STI571 treatment induces *Rag1*, *Ig κ* GLT, and *Irf4* expression as assessed by quantitative RT-PCR in B5 and A2 clones. (C) D_{DFL16.1} → J_{H1} CJs assessed in A2 and B5 clones transfected with empty (E) or *Rag2* (R) expression constructs and the Mb1 loading control. (D, top) Treatment timeline in PA112.2 cells. (Bottom) Quantitative RT-PCR for *AID* and *Rag2* expression. (E) Semiquantitative RT-PCR for PST $\gamma 2b$ and ε or *Hprt*. (F) V _{$\kappa 6-23$} → J _{$\kappa 1$} CJs and Mb1 assessed by semiquantitative PCR.

PA112.2 cells (strain 129; L κ , germline) were exposed to CSR inducers for 48 h, and switching was activated as indicated by increased expression of *AID* and PSTs $\gamma 2b$ or ε , which persisted for 8 h following withdrawal of the CSR inducers (Fig. 2D,E). In cells exposed to STI571, *AID* diminished, and *Rag2* concomitantly increased, accompanied by active V _{$\kappa 6-23$} -J _{$\kappa 1$} CJ formation (Fig. 2D,F) with appropriate junctional diversity (Supplemental Fig. S12). CSR induction had no effect on subsequent generation of V _{$\kappa 6-23$} -J _{$\kappa 1$} CJs (Supplemental Fig. S13). Active PST expression following STI571 treatment indicates that populations of switched cells are fully capable of V(D)J joining (Fig. 2D,E). We conclude that B cells expressing BCR with secondary isotypes may emerge directly from the BM. Intriguingly, the CSR or V(D)J gene programs can be alternately expressed in response to specific inducers during early B-cell ontogeny.

The pattern of CSR in pro-B cells is skewed toward IgG2b and IgE and is atypical of mature B cells. Germline transcription in activated mature B cells is regulated by the spatial organization of the *Igh* locus through long-range chromatin contacts between the E μ and 3'E α enhancers and GLT promoters (Supplemental Fig. S14A; Wuerffel et al. 2007; Sellars et al. 2009). Could $\gamma 3$: $\gamma 1$ contacts in a developmental stage-specific chromatin structure restrict CSR in pro-B cells (Supplemental Fig. S14B)? Using the chromosome conformation capture (3C) carbon copy (5C) method for unbiased detection of chromatin contacts (Dostie et al. 2006), we simultaneously examined a matrix of 12,656 possible looping interactions in parallel across the entire *Igh* locus and focused here on the 220-kb C_H domain spanning E μ and

3'E α (Supplemental Material; Supplemental Fig. S15; Supplemental Tables S2, S3). We detected abundant $\gamma 3:\gamma 1$ chromatin interactions (Fig. 3, orange circle), indicating a compact conformation in *Rag2*^{-/-} pro-B cells that is absent in resting splenic B cells (Fig. 3). We examined the log₂ ratio pro-B/resting B 5C interactions (represented by red [pro-B] and blue [resting B] in Fig. 3) and confirmed that $\gamma 3:\gamma 1$ interactions are elevated in pro-B cells (Fig. 3). We found that E μ : $\gamma 2b-\epsilon$ and E μ : $\gamma 3-\gamma 1$ contacts are associated with pro-B and resting B cells, respectively, in accord with the pattern of potential GLT expression and CSR. In contrast, few long-range interactions were detected for a gene desert region on chromosome 5 (Supplemental Fig. S16).

3C studies confirmed abundant $\gamma 3:\gamma 1a$ (1.7-fold, $P = 0.053$) and $\gamma 3:\gamma 1b$ (twofold, $P = 0.01$) chromatin contacts in *Rag2*^{-/-} pro-B cells as compared with resting B cells or ConA-activated splenic T cells, where $\gamma 1a$ and $\gamma 1b$ are opposite ends of Hind III fragment C (Fig. 4A,B). *Rag2*^{-/-} pro-B cells and the 445.3 pro-B-cell line display a similar profile of $\gamma 3:\gamma 1$ interactions, indicating that in early B cells, a compacted higher-order chromatin structure involving $\gamma 3:\gamma 1$ may limit that accessibility to the CSR machinery (Supplemental Fig. S17). In contrast, $\gamma 3:\gamma 2b$ contacts observed in both *Rag2*^{-/-} pro-B cells and wild-type resting B cells are similar to each other and may be related to a spatial organization that is absent in T cells (Fig. 4B).

Because interaction of distal Igh enhancers with the targeted *C_H* locus is essential to bring participating S regions into close proximity (Wuerffel et al. 2007), we examined *Rag2*^{-/-} pro-B and 445.3 cells for these chromatin contacts in 3C assays. 3'E α :E μ (H-A) interactions in *Rag2*^{-/-} pro-B cells and resting splenic B cells are similarly abundant and significantly higher than in ConA-stimulated splenic T cells (Fig. 4C, left panel). When CSR is induced in LPS+IL4-activated B cells,

3'E α :E μ (H-A) interactions become elevated relative to resting B cells (Fig. 4C, left panel). Notably, when the 445.3 pro-B-cell line is activated with LPS+CD40L to initiate CSR, 3'E α :E μ (H-A) ($P < 0.006$) and hs3b,4: $\gamma 2b$ ($P < 0.002$) interactions significantly increase, whereas $\gamma 3:3'E\alpha$ interactions remain unchanged (Fig. 4C, right panel). Thus, our results demonstrate a unique three-dimensional chromatin structure in pro-B cells that supports isotype-specific CSR and appears to preclude $\gamma 3$ and $\gamma 1$ GLT expression and CSR in a developmental stage-specific fashion (Supplemental Fig. S14B).

Our findings establish that pro-B cells in the BM can undergo robust CSR, biased toward IgG2b and IgE, in response to specific stimuli followed by V(D)J recombination. Thus, pro-B cells that are delayed in the BM by genetic predisposition (as in Omenn syndrome) or environmental cues have the capacity to undergo CSR in response to bacterial infections and to eventually populate the periphery following V(D)J joining. Two additional conclusions flow from our findings. The origin of DNA damage reminiscent of both RAG and AID activity in some human leukemias (Tsai et al. 2008) has been difficult to explain. This footprint of DNA damage in leukemia may be related to coexpression of AID and RAG1/2 that we observed in pro-B cells induced to switch. Most notably, alternating expression of CSR versus V(D)J joining in early B cells reveals an unanticipated flexibility of these gene programs, with implications for our understanding of developmental and lineage commitment.

Materials and methods

In brief, mice were handled according to institutional and National Institutes of Health (NIH) guidelines. Standard protocols were used for cell culture, quantitative PCR, RT-PCR, DC-PCR, CT-PCR, retroviral transductions, statistical analyses, and switch, V κ -J κ , and D H -J H junction

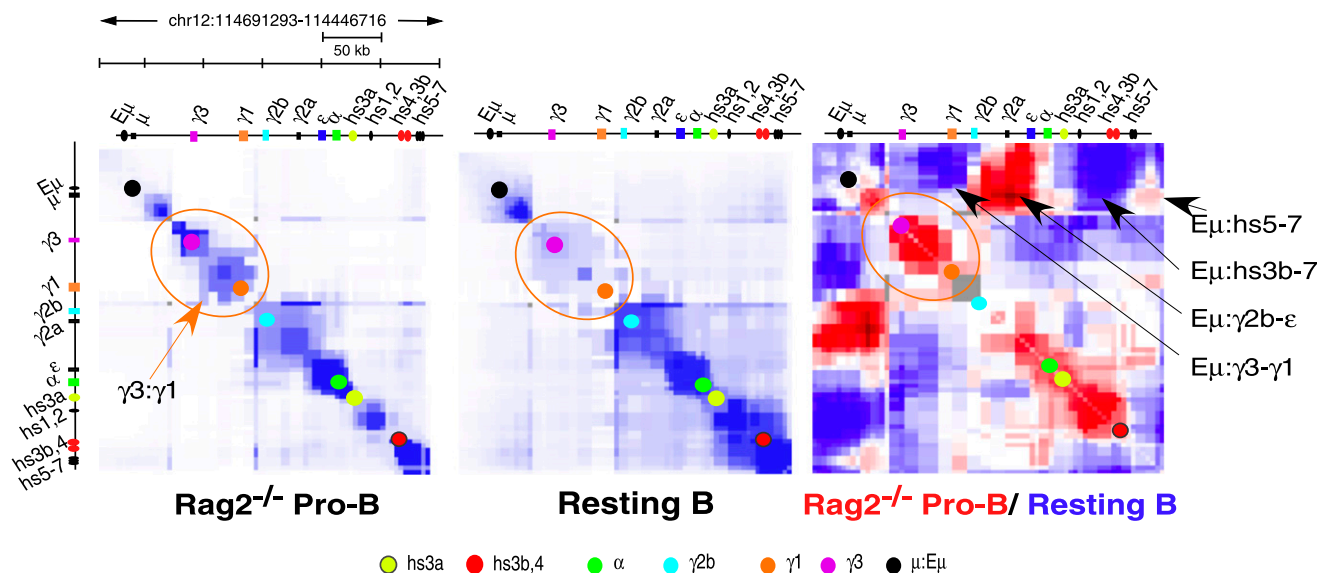


Figure 3. A developmentally stage-specific chromatin configuration of the *Igh* locus. 5C analyses of the *Igh* locus in *Rag2*^{-/-} pro-B cells and resting splenic B cells are represented in heat maps of 5C data (bin size 30 kb; step size 3.0 kb). Interaction frequencies are plotted against genomic distance. Blue intensity represents the contact frequency between genomic coordinates. The diagonal represents frequent proximal intrachromosomal interactions along the linear *Igh* locus. 5C signals off the diagonal indicate looping interactions. (Right panel) Log₂ ratio of pro-B/resting B 5C interactions where pro-B (red) and resting B (blue) contact.

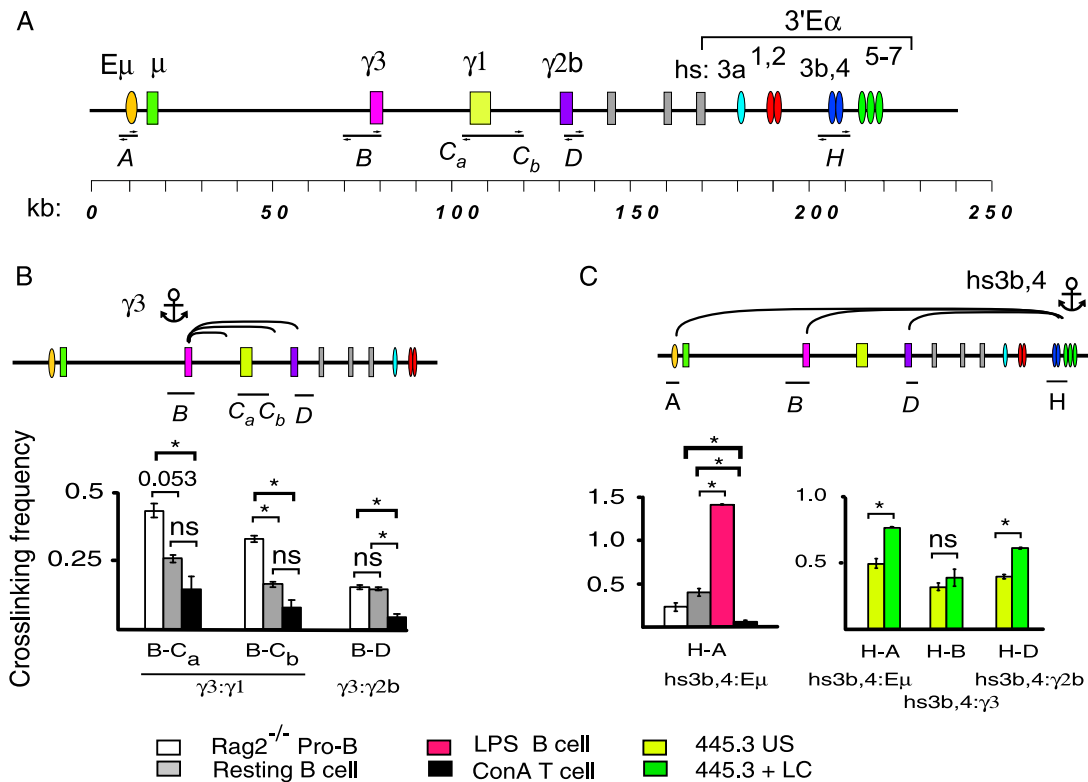


Figure 4. Absence of IgG3 and IgG1 CSR in pro-B cells is related to looping interactions between the $\gamma 3$ and $\gamma 1$ loci. (A) A segment of the *Igh* locus spanning 220 kb (shown at the top) in which 3C HindIII restriction fragments (A, B, C, D, and H) are shown. (B,C) 3C assays using chromatin from *Rag2*^{-/-} pro-B cells, unstimulated (US) or LPS+CD40L (LC)-stimulated 445.3 cells, wild-type (WT) resting splenic B cells, or ConA-stimulated T cells. (*) $P \leq 0.05$; (ns) Not significant. (B) 3C assays anchored at $\gamma 3$ were analyzed for interactions with $\gamma 1$ or $\gamma 2b$. (C) Hs3b,4 (3'E α) anchor was analyzed for interactions with $E\mu$, $\gamma 3$, or $\gamma 2b$.

cloning. Abelson-MuLV transformed pro-B-cell or pre-B-cell lines were kindly provided by Dr. B. Sleckman (Washington University, St. Louis). 3C assays were carried out (Wuerffel et al. 2007) in combination with 5'FAM/ 3'BHQ1-modified probes. 5C primers were designed using online tools (<http://my5C.umassmed.edu>). 5C library construction was performed as described (Dostie et al. 2006). Additional methods are available in the Supplemental Material.

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