UNIVERSITY OF CALIFORNIA, SAN DIEGO

Association of E47 with Immunoglobulin Kappa Light Chain Enhancers in B Lymphocytes and Its Implication in Allelic Exclusion

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by

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ABSTRACT OF THE THESIS

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Professor Cornelis Murre, Chair

The production of diverse antibodies in B cells is the end result of the combinatorial rearrangement of immunoglobulin genes. However, given the choice of two immunoglobulin heavy or light chain alleles, a functional rearrangement occurs only on one allele at a given time, and B cells subsequently express surface receptors with a single specificity, a phenomenon termed allelic exclusion. Although it has been a subject of intensive research, the exact mechanisms for the establishment of allelic exclusion are yet to be fully understood. In this study, we provide evidence suggesting that E47 might be involved in the differential accessibility of two immunoglobulin

kappa light chain alleles. Using the combination of biarsenical-tetracysteine (TC) labeling system and 3D fluorescence in situ hybridization (3D FISH) to visualize E47 and the Igκ locus, we demonstrate that the association of E47 with the Igκ intronic/3′ enhancers in pro-B and pre-B cells is predominantly monoallelic. Furthermore, the analysis of the spatial distances between the distal Vκ region and the Igκ enhancers among pro-B and pre-B cells showing monoallelic E47 binding revealed that the E47-bound Igκ alleles are in a more compact state than the non-E47-bound Igκ alleles. In addition, E47 association correlates with a more central subnuclear localization of the Igκ locus.

Hence, our findings suggest that the monoallelic interaction of trans-acting factors such as E47 and cis-acting regulatory DNA elements might contribute to the establishment of allelic exclusion at the Igκ locus by creating differential locus environments. These findings may provide valuable insight into the potential universal mechanism for allelic exclusion during lymphocyte development in general.

Introduction

B lymphocytes are derived from hematopoietic stem cells (HSC) in the bone marrow through a series of highly regulated developmental stages (Fig. 1). Once common lymphoid progenitors (CLPs) become committed to the B cell fate, they undergo immunoglobulin heavy chain (IgH) rearrangement. This allows for the expression of a pre-B cell receptor (pre-BCR) whose subsequent signaling serves as the first developmental checkpoint and promotes the expansion and differentiation of pre-B cells, followed by the rearrangement of immunoglobulin light chain (IgL) genes. The expression of a B-cell receptor (BCR) serves as the second developmental checkpoint, ultimately leading to the generation of immature B cells. Then the cells migrate to peripheral lymphoid tissues in which they become mature B cells and undergo Ig isotype switching and somatic hypermutation upon antigen recognition.

The developmental progression of B cells is partly mediated by the coordinated action of lineage- and stage-specific transcription factors. One class of transcription factors known as basic helix-loop-helix (bHLH) proteins is of particular importance. Class I bHLH proteins, also known as E proteins, are transcription factors that

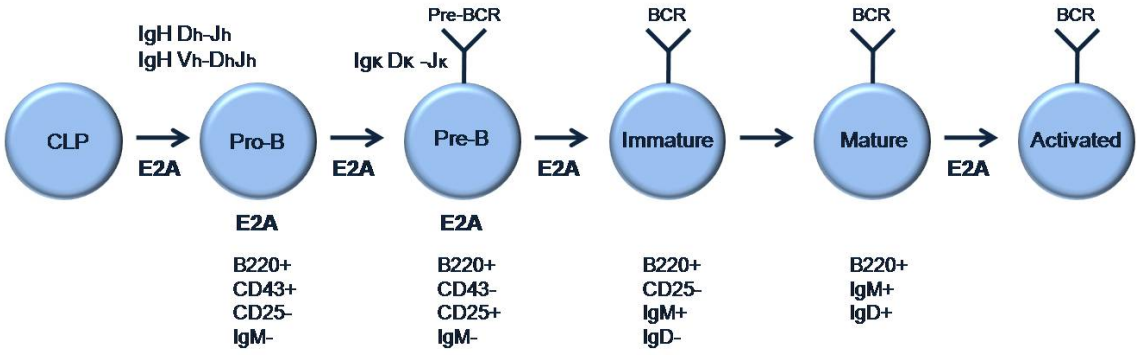

Figure 1. Schematic diagram of B cell development. Shown is the progression of B cell development and surface markers expressed at the corresponding stages. The developmental stages in which E2A is known to function are noted with "E2A". (CLP = common lymphoid progenitor)

specifically bind to the canonical “E box” sequence CANNTG[28], and E boxes are found in the promoters and enhancers of various lymphoid lineage-specific genes[19]. The mammalian E protein family includes E12, E47, E2-2, and HEB (HeLa E-box binding protein)[28], all of which share a HLH dimerization domain and a basic region that mediates DNA binding[44]. In addition, E proteins have two highly conserved regions, termed AD1 and AD2, that allow for the recruitment of co-activator or co-repressor complexes[43]. Among the E protein family members, E12 and E47, collectively known as E2A proteins, are encoded by a single gene, *Tcfe2a*, and arise from alternative splicing of an exon encoding the bHLH region[43]. In developing B cells, E12 is generally found as a heterodimer with E47 or other bHLH proteins, whereas E47 function mainly as a homodimer[4,19].

E2A proteins are highly expressed in developing B cells, and their important functions in B lineage specification and differentiation have been demonstrated in the previous studies. First, E2A is required for the initiation of B cell development in the bone marrow as E2A-deficient mice show a complete developmental block at the pre-pro-B cell stage, prior to the initiation of immunoglobulin heavy chain gene rearrangement[43]. Additionally, E2A is crucial for the progression of B cell development in the context of immunoglobulin gene rearrangement. It has been demonstrated that the over-expression of E47 in a pre-T cell line activates germline Igμ heavy chain transcription and induces Dh-Jh rearrangement[60]. Furthermore, the ectopic expression of E2A along with RAG proteins in non-lymphoid cells such as embryonic kidney cell line BOSC23 is sufficient to activate germline transcription and Igκ VκJκ gene rearrangement[16,55]. Thus, these findings indicate that E2A proteins have potential to

promote immunoglobulin gene rearrangement and key regulators in the process.

The nature of interaction between E2A and immunoglobulin loci in the context of rearrangement can be further understood by studying the overall structure of immunoglobulin loci and regulatory elements found within them. The murine immunoglobulin kappa (Igκ) light chain locus has been particularly well studied in this regard. The murine Igκ locus is located on chromosome 6 and approximately 3.4 Mb long[56] (Fig. 2). There are about 140 Vκ gene segments, which start about 20kb upstream of five Jκ gene segment cluster, and a single constant-region exon (Cκ) roughly 2.5 kb downstream of the Jκ gene segment cluster[25,26].

Within the Igκ locus, there are two well-characterized transcriptional enhancers: κ intronic enhancer (Eiκ) within the J-C intron[48,49] and 3´ enhancer (3´Eκ)[39] located 9 kb downstream of Cκ[61]. Eiκ contains three E boxes: κE1, κE2, and κE3. E2A proteins are known to bind to κE2 and κE1 to a lesser extent, but not κE3[23]. Previous studies have shown the importance of these enhancers, particularly E boxes found within them, for Igκ locus activation and gene rearrangement. It has been shown that mutations introduced to either κE1 or κE2 differentially reduce the efficiency of κ rearrangement, and the effect of the simultaneous mutations of both E boxes is equivalent to that from

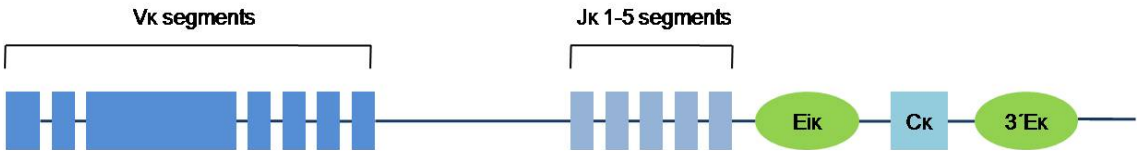

Figure 2. Schematic representation of murine germline immunoglobulin kappa (Igκ) light chain locus. Cκ, Eiκ, and 3´Eκ denote κ-constant-region exon, κ intronic enhancer, and k 3´ enhancer, respectively. The diagram is not drawn to scale. *The number of κ gene segments shown are for representation only, and do not reflect the actual number.

the deletion of the entire intronic enhancer[23]. Therefore, understanding the importance of E boxes within the Igκ enhancers in Igκ gene rearrangement, it would be valuable to study the binding of E47 to the Igκ enhancers and its effect on Igκ locus configuration.

If the nature of association between a protein and DNA regulatory elements were to be understood, immunofluorescence in conjunction with FISH (fluorescence in situ hybridization) can be used to characterize the interaction. In studying the localization and dynamics of a protein in living cells, the fusion of genetically encoded fluorescent proteins such as GFP to the protein of interest has been a valuable tool. However, GFP is potentially perturbative to the protein of interest due to its large size (238 amino acids), and therefore has potential to affect the activity, localization, and conformation of its fusion partner[1,3,21]. An alternative to GFP or other conventional fluorescent proteins is biarsenical-tetracysteine (TC) labeling system. Biarsenical-TC labeling is used to fluorescently label recombinant proteins by genetically attaching a small motif (6 to 20 residues) containing the sequence -Cys-Cys-Pro-Gly-Cys-Cys to the protein of interest and exposing cells to a membrane-permeant nonfluorescent biarsenical derivative of resorufin, ReAsH[14] (Fig. 3). Upon the addition of ReAsH, each arsenic atom in ReAsH forms a reversible covalent bond with a pair of cysteine residues[65]. ReAsH has the excitation and emission maxima at 593 and 608 nm, respectively[14], and the use of small competitive dithiol antidotes such as 1, 2-ethanedithiol (EDT) minimizes non-specific binding and toxicity of arsenic compounds to endogenous proteins containing cysteine pairs[15].

The biarsenical-TC labeling system provides unique capabilities compared to other conventional fluorescent proteins. First of all, its main advantage is that, due to

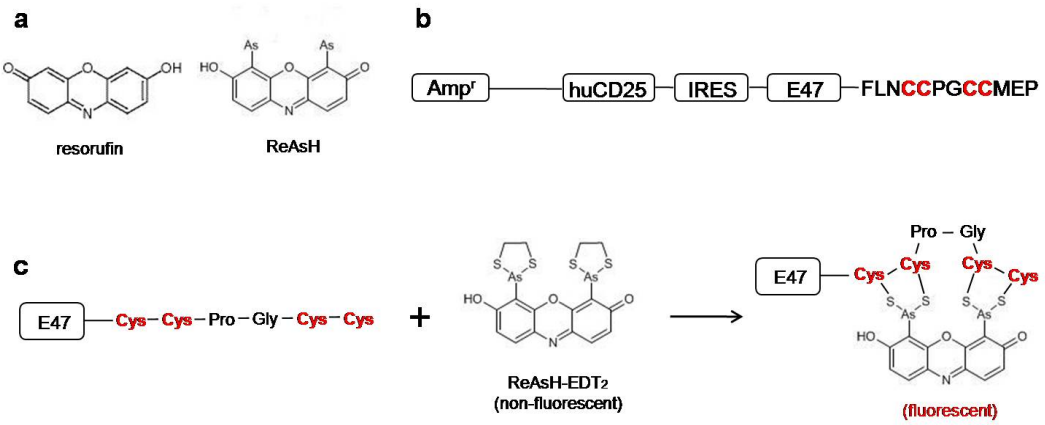

Figure 3. ReAsH labeling of tetracysteine-tagged E47. (a) Chemical structures of resorufin and ReAsH. (b) Design of MinVTAC E47TC construct. (c) Schematic diagram of ReAsH labeling

the small size of the tag, the tetracysteine motif is much less likely to perturb the function of the protein of interest[34,35]. ReAsH also displays very little fluorescence when complexed with EDT, but becomes brightly fluorescent upon binding to a TC motif[14]. In addition, TC-tagged proteins are detectable immediately after translation[35], whereas the folding of fluorescent proteins such as GFP is far from instantaneous[1]. Thus, using the biarsenical-TC labeling system may provide a novel and improved approach to visualize the interaction of a protein and its DNA-binding site.

In studying the binding of E47 to the Igκ enhancers in pre-B cells, IL-7 withdrawal can be used to differentiate pro-B to pre-B cells in vitro. IL-7 is a stromal cell-derived cytokine and necessary for the proliferation and differentiation of committed B cell progenitors in vitro[10,11,63]. Previous studies have shown that B cell precursors grown in the presence of IL-7 undergo IgH VDJ rearrangement, retain IgL locus in germline configuration, and do not express surface IgM[6,11,53,54]. Upon IL-7 removal from the culture medium, there is an increase in RAG expression, IgL gene rearrangement, and expression of surface IgM[38]. However, upon IL-7 withdrawal, pro-B

cells undergo cell cycle arrest and subsequently cell death most likely due to an elevated Bax to Bcl-2 ratio in the absence of IL-7 signaling[32]. Therefore, IL-7 withdrawal alone would not allow for studying these cells through differentiation. However, it is known that the over-expression of an anti-apoptotic protein, Bcl-2, promotes the viability of cytokine-dependent cells upon cytokine withdrawal. For instance, it has been shown that the over-expression of Bcl-2 in IL-3-dependent pro-B cell lines and pro-myeloid cell lines prolongs cell survival following IL-3 withdrawal[9]. Therefore, in order to circumvent IL-7 withdrawal-induced apoptosis during the course of in vitro pro-B to pre-B cell differentiation, we over-expressed Bcl-2 in pro-B cells prior to IL-7 withdrawal.

Using the combination of the ReAsH-TC labeling system and FISH, we visualized the association of E47 with the Igκ enhancers in pre-B cells, and understanding the nature of the interaction has an important implication in allelic exclusion. Allelic exclusion, one of the most intriguing, yet enigmatic biological phenomena, is the process by which B and T lymphocytes express a single antigen receptor, given the choice of two alleles for each gene. However, the underlying assumption of this definition is that the rearrangement of Ig genes occurs on a single allele at a given time. Furthermore, a broader implication of allelic exclusion is how the rearrangement of Ig and TCR loci are controlled in different developmental stages or lineages. Despite new findings, the exact mechanisms behind allelic exclusion are far from completely elucidated. However, many studies allowed for the construction of a working model. One of the important underlying themes in allelic exclusion is negative feedback inhibition[51]. When there is a productive rearrangement on the first allele and

subsequent expression of a functional receptor on the cell surface, RAG expression is down-regulated, thus preventing rearrangement on the second allele[17]. However, if the rearrangement on the first allele is not productive, RAG expression is not down-regulated, and therefore the second allele can undergo rearrangement[17]. While this idea is important for the maintenance of allelic exclusion, it does not account for differential rearrangement. Since RAG proteins are made available to all immunoglobulin loci for potential rearrangement, it has been suggested that the control mechanism is probably differential locus accessibility[41]. Therefore, another theme that may contribute to the establishment of allelic exclusion is differential chromatin changes. There are two cellular processes that could potentially mediate these changes: DNA demethylation and histone acetylation. It has been demonstrated that DNA demethylation correlates with locus accessibility and gene activation, and that the demethylation of κ genes is monoallelic in κ+ B cells[40,42]. In addition, histone acetylation can also bring about increased locus accessibility as it has been shown that increased histone acetylation correlates with promotion of V_h-D_hJ_h rearrangement[24]. The prevalent idea in DNA demethylation and histone acetylation is that differential chromatin changes are mediated by local cis-acting elements[17,42]. Hence, these ideas suggest that allelic exclusion is probably established at the chromatin level and in part mediated by trans-acting factors that bind to such cis-acting elements.

The potential role of E2A in allelic exclusion can be evaluated on the ground of differential locus environments mediated by its association with the Igκ enhancers. Locus compaction and subnuclear localization relative to nuclear periphery can serve as indicators of relative locus accessibility. It has been shown that in pro-B cells IgH loci

show significantly higher frequencies of locus compaction compared to T cells, reflecting a locus state that is more conducive to rearrangement[57]. Also, it is well-known that, when regulatory elements separated by relatively long distances are brought into physical proximity, this leads to an open chromatin domain and activate transcription and other cellular processes[8,47,64]. This is especially relevant for Igκ locus because its large size (>3Mb) poses a biophysical barrier for potential gene rearrangement. Thus, based on these ideas, the state of locus compaction can reflect the overall accessibility of a given locus.

Another way to assess locus accessibility is subnuclear localization. Nuclear periphery is known to be a transcriptionally repressive environment[27]. One study has shown that IgH and IgL loci are preferentially located near nuclear periphery in hematopoietic progenitors and pro-T cells in which such perinuclear localization correlates with limited rearrangement, whereas these Ig loci are centrally located in Pro-B cells[27,58]. Also, unopened alleles of immunoglobulin loci are known to be localized to the nuclear periphery after B cells are induced to proliferate[62].

Therefore, if the correlation between E47 association and locus compaction/central subnuclear positioning were to be made, this might provide additional evidence that E47 can potentially play a role in allelic exclusion. Based on the previous findings that E47 has potential to control locus accessibility for immunoglobulin gene rearrangement during B cell development, its interaction with Igκ enhancers is likely to be of importance. To address how E47 might contribute to the establishment of allelic exclusion in pre-B cells, we employed the ReAsH-tetracysteine (TC) labeling system and 3D fluorescence in situ hybridization (3D FISH) to visualize

the association between E47 and the Igκ intronic/3´ enhancers. In this study, we demonstrate that E47 associates with the Igκ intronic/3´ enhancer region predominantly monoallelically in pro-B and pre-B cells. Furthermore, E47 binding to the Igκ enhancers correlates with locus compaction and central subnuclear localization in pro-B cells and pre-B cells. Thus, these findings indicate that the monoallelic nature of association between trans-acting factors such as E47 and cis-acting regulatory elements is likely to result in differential locus accessibility, and this mechanism may prove to be a crucial basis for the establishment of allelic exclusion.

Results

ReAsH signals observed upon labeling with ReAsH are tetracysteine motif-specific

We have previously generated the plasmid construct, MinVTAC E47TC, containing E47 cDNA with a tetracysteine (TC) tag to allow for the expression of TC-tagged E47 in pre-B cells. In order to test if TC-tagged E47 is expressed and readily detected with ReAsH above the background level, various control stainings were performed (Fig. 4a-e). When non-transduced Pro-B cells were stained with anti-lamin antibodies, no red fluorescence was observed as expected (Fig. 4a). When non-transduced and MinVTAC E47-transduced pro-B cells were stained with ReAsH, there was little fluorescence observed in the nucleus (Fig. 4b-c). However, there was some background fluorescence detected in the cytoplasm, and this is most likely due to thiol-independent hydrophobic interactions between ReAsH and cellular components[20] as increasing 1, 2-ethanedithiol (EDT), which helps to reduce background staining by competitively binding to ReAsH, up to a millimolar concentration did not significantly reduce such non-specific staining in the previous optimization experiments (data not shown). On the contrary, upon labeling with ReAsH, MinVTAC E47TC-transduced pro-B cells showed many distinct, concentrated puncta of ReAsH signals in the nucleus (Fig. 4d). This indicated that TC-tagged E47 was properly localized to the nucleus. Thus, these observations demonstrated that the fluorescence observed with ReAsH staining upon the introduction of recombinant E47 was tetracysteine motif-specific.

In addition, this study was the first attempt to visualize a DNA-protein interaction using the combination of the biarsenical-TC labeling system and FISH.

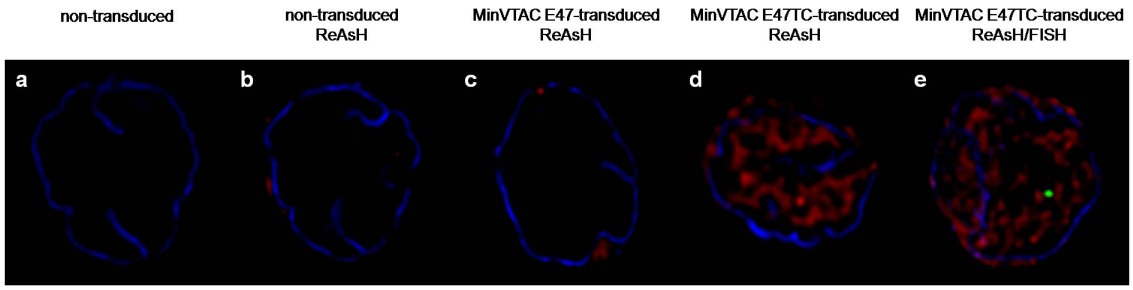

Figure 4. ReAsH signals are tetracysteine motif-specific. (a-e) The staining conditions are denoted above each image. The nuclear membrane is stained with anti-lamin antibodies and shown in blue. Red and green are E47TC (labeled with ReAsH) and BAC RP24-279F16 (labeled with Alexa-488), respectively. *All cells shown are cultured pro-B cells.

Because some of the FISH procedures involve the use of HCl and high temperature incubations, all of which can potentially degrade proteins and interfere with the detection of TC-tagged proteins by ReAsH, we also tested the compatibility of these two labeling techniques. To this end, the stainings between ReAsH only-treated, MinVTAC E47TC-transduced pro-B cells and ReAsH/FISH-treated, MinVTAC E47TC-transduced pro-B cells were compared for ReAsH fluorescence (Fig. 4d-e). The qualitative assessment of distribution and abundance of ReAsH signals was done in over 30 cells from each group (data not shown), and it did not differ significantly between two groups. Therefore, this indicated that ReAsH-TC labeling system and FISH are compatible.

Pro-B cells display pre-B cell surface phenotypes following IL-7 withdrawal

It is known that, following the removal of IL-7 from the culture medium, pro-B cells up-regulate RAG expression, undergo IgL gene rarrangement, and display other pre-B cell traits[38]. Furthermore, the over-expression of Bcl-2 promotes the survival of cytokine-dependent cells upon cytokine withdrawal[9]. In order to differentiate pro-B

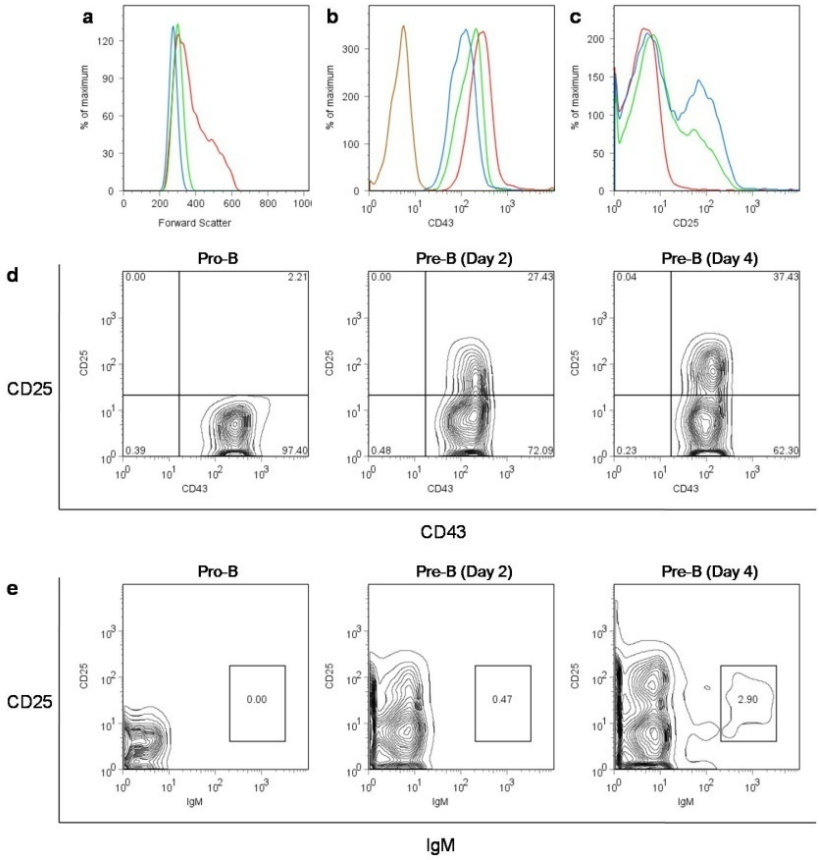

Figure 5. Pro-B cells display pre-B cell surface phenotypes upon IL-7 withdrawal. (a-c) Flow cytometry on cell size, CD43 and CD25 expression (Red: Pro-B, Green: Pre-B (Day2), Blue: Pre-B (Day-4), and Brown: Pro-B (unstained)). (d) Contour plot of CD43 and CD25 expression at different time points following IL-7 withdrawal. (e) Contour plot of CD25 and IgM expression at different time points following IL- withdrawal.

cells to pre-B cells in vitro and to promote cell survival, we transduced pro-B cells with a Bcl-2 construct prior to IL-7 withdrawal. During the course of IL-7 withdrawal, we monitored cell size based on the forward scatter and surface phenotypes to evaluate the extent of pro-B to pre-B cell differentiation in vitro. A dramatic decrease in cell size was observed 2 days following IL-7 withdrawal, and there observed a further reduction on day 4 post IL-7 withdrawal (Fig. 5a). This observation was consistent with the fact that large, cycling pro-B cells become smaller upon differentiating into non-cycling pre-B cells[12,22,30.]

We also measured the level of CD43 expression since it is expressed on pro-B cells, but not on pre-B cells. Flow cytometry data revealed that the expression of CD43 was down-regulated following IL-7 withdrawal (Fig. 5b, d). Although the cells remained $CD43^+$ even 4 days following IL-7 withdrawal, $CD43^-$ populations were usually observed 6 days after IL-7 removal (data not shown). On the contrary, the expression of CD25, which is expressed only on pre-B cells, followed the opposite pattern. Prior to IL-7 withdrawal, almost all cells were $CD25^-$ (Fig. 5c, d). However, even on day 2 post IL-7 withdrawal, there was a substantial proportion (~27%) of $CD25^+$ cell, and the proportion of cells expressing CD25 increased to ~37% by day 4. Also, the CD25 expression was further up-regulated at later time points (data not shown). Lastly, we also checked if these pro-B cells could differentiate beyond the pre-B cell stage in vitro. By day 4 post IL-7 withdrawal, a small fraction of IgM^+ cells started to appear (Fig. 5e), and as seen with CD25 expression, a further up-regulation was observed at later time points (data not shown). Thus, these data indicated that pro-B cells display pre-B cell surface phenotypes and, in general, mature traits upon IL-7 withdrawal, and that this system can be used to study the Igκ locus in the context of pro-B to pre-B cell differentiation.

Igκ loci are more compact and centrally localized in pro-B and pre-B cells than A12 cells.

Previously it has been shown that IgH loci show more compaction and central subnuclear localization in pro-B cells than T cells, and these findings demonstrated the differential regulation of IgH in different lineages[57]. Thus, before evaluating any

specific interaction between E47 and the Igκ locus, we measured the locus compaction state and subnuclear localization to see how Igκ loci are organized overall in different developmental stages or lineages. By characterizing its organization, we wanted to see whether or not it is conducive to gene rearrangement. The configuration of the Igκ locus in pro-B and pre-B cells was determined by measuring the spatial distance between two regions found within the Igκ locus by 3D FISH. Two different BAC probes were used for this purpose: BAC RP23-234A12 probe targeting the distal Vκ region and BAC RP24-279F16 targeting the Igκ intronic/3´ enhancer region (Fig. 6a). In addition, A12 cells, which are 1.F9 E2A-deficient thymoma line transduced with retrovirus encoding Bcl-2[13], were used as a non-B cell lineage control.

Our first observation was that the Igκ loci were already compact in pro-B cells and remained compact throughout pre-B cell differentiation (Fig. 6b, d). However, the Igκ loci were relatively non-compact in A12 cells (Fig. 6c-d). When the average spatial distances between the distal Vκ region and enhancer region in pro-B and pre-B cells were compared with those from A12 cells, the differences were statistically significant in all cases as denoted by the *p* values (Fig, 6d; Tab. 1). These results demonstrated that the Igκ loci are in a compact state in pro-B and pre-B cells, reflecting overall locus accessibility, and the locus compaction state is differentially regulated in B and T cells.

As another indicator of locus accessibility, we also assessed subnuclear localization by measuring the spatial distances from either the distal Vκ region or the enhancer region to nuclear membrane. In both pro-B and pre-B cells, the Igκ loci are centrally localized in comparison with A12 cells (Fig. 6e, f; Tab 1.). Furthermore, we

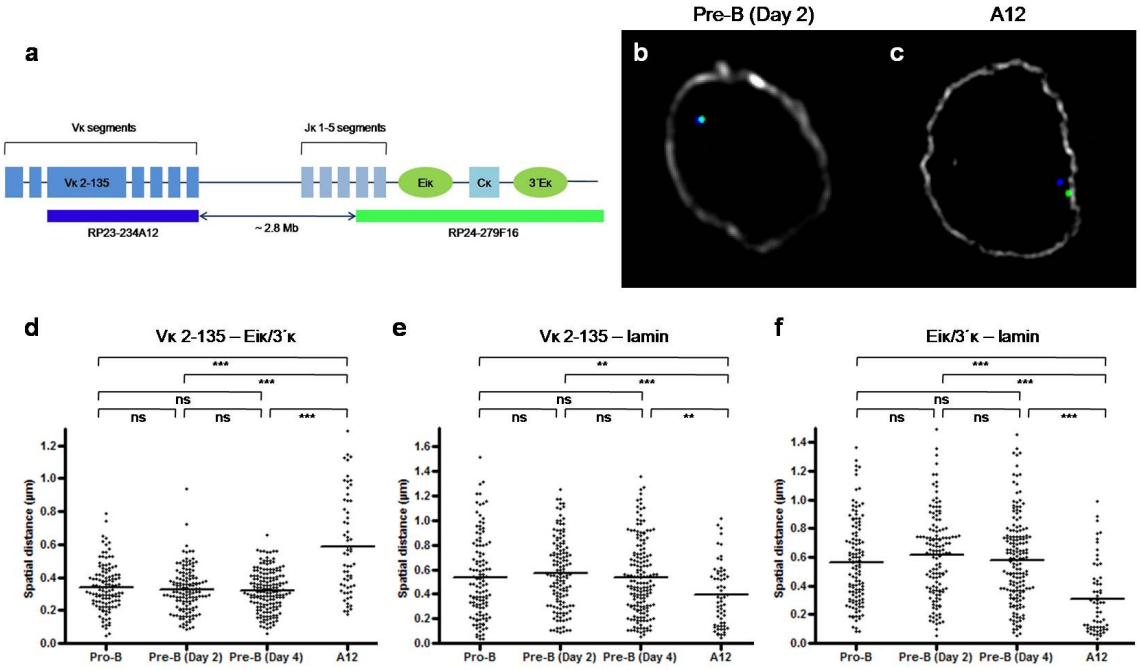

Figure 6. Overall Igκ locus configuration and subnuclear localization in pro-B, pre-B, and A12 cells (a) The murine Igκ locus and positions of the two BAC probes are indicated (not drawn to scale). The colors of the probes are shown as follows: RP23-234A12 (blue) and RP24-279F16 (green). (b) Igκ locus compaction observed in pre-B cells (Day 2 post IL-7 withdrawal). (c) Igκ locus de-compaction observed in A12 cells. (d) Scatter-plots of the spatial distances separating the distal Vκ region and intronic/3´ enhancer region. (e) Scatter-plots of the spatial distances between Vκ 2-135 and nuclear membrane. (f) Scatter-plots of the spatial distances between the Eiκ/3´ enhancer region and nuclear membrane. Black line represents the average distance within the group. Significant differences are denoted as following: *** ($p < 0.001$; extremely significant), ** ($p < 0.01$; highly significant), * ($p < 0.05$; significant), and ns (non-significant). Downward brackets indicate data groups taken for the pair-wise t test.

Table 1. Spatial distances separating Vκ 2-135 and Eiκ/3´Eκ, Vκ 2-135 and nuclear membrane, and Eiκ/3´Eκ and nuclear membrane in pro-B, pre-B, and A12 cells. Shown are average spatial distances ± SD expressed in µm. *p* values (*** and **) were calculated in comparison to A12 cells. (N = the number of cells taken for analysis from each group).

	Pro-B	Pre-B (Day 2)	Pre-B (Day 4)	A12
N	60	68	88	30
Vκ 2-135 – Eiκ/3´Eκ	0.34 ± 0.14 (***)	0.32 ± 0.13 (***)	0.32 ± 0.12 (***)	0.59 ± 0.30
Vκ 2-135 – lamin	0.53 ± 0.33 (**)	0.57 ± 0.29 (***)	0.53 ± 0.30 (**)	0.39 ± 0.26
Eiκ/3´Eκ – lamin	0.57 ± 0.30 (***)	0.61 ± 0.29 (***)	0.58 ± 0.30 (***)	0.31 ± 0.25

observed that the Igκ enhancer region is farther away from nuclear periphery in pro-B cells, whereas it is closer to nuclear membrane in A12 cells. This resulted in more pronounced differential subnuclear positions of the enhancer region relative to nuclear lamin in pro-/pre-B cells and A12 cells (Fig. 6f). Again the statistical significance was measured based on the *p* values (Tab. 1). Overall, these data indicated that the Igk loci are in a relatively open configuration in pro-B and pre-B cells, and that the Igκ loci already become accessible in pro-B cells. These results were consistent with our findings on locus compaction state.

E47 associates with the Igκ intronic/3´ enhancers mostly monoallelically in pro-B and pre-B cells

Our previous data compared the configuration of Igκ loci among different developmental stages and lineages. However, another important question to ask would be how Igk loci are controlled within the same cell. The existing model of allelic exclusion suggests that differential locus accessibility may determine allelic choice for rearrangement via DNA demethylation and histone acetylation[24,41,42.] Since such chromatin changes are known to be mediated by the interaction between trans-acting factors and cis-acting elements, we looked at the nature of association between E47 and the Igκ intronic/3´ enhancers to study its implication. We employed the ReAsH-tetracysteine (TC) labeling system and 3D FISH to visualize the association in pro-B and in vitro differentiated pre-B cells using IL-7 withdrawal. Then the proportion of cells showing monoallelic, biallelic, or no colocalization of E47 with the Igκ enhancers was noted.

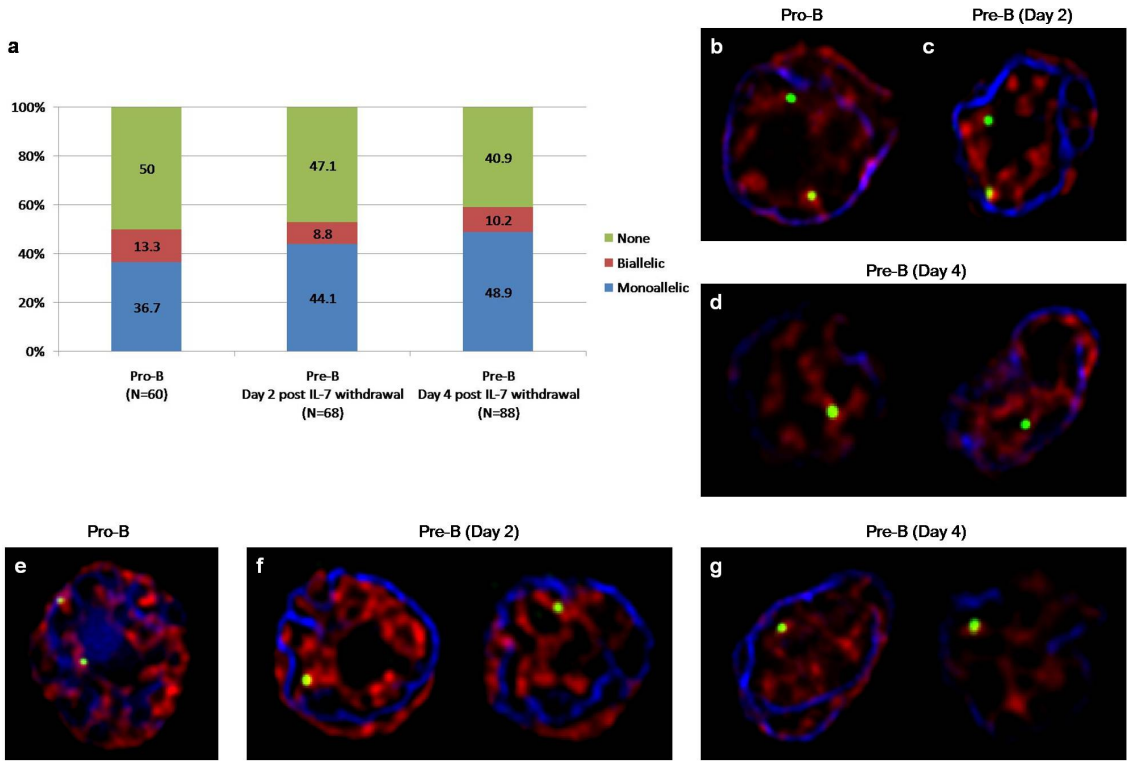

Figure 7. The association of E47 with the Igκ intronic/3´ enhancers is predominantly monoallelic. (a) Proportion of cells in which monoallelic, biallelic, or no colocalization of E47 with the Igκ intronic/3´ enhancers is observed (N = the number of cells analyzed from each group). (b-d) Examples of monoallelic colocalization. (e-g) Examples of biallelic colocalization. Note: red (E47TC), green (BAC RP24-279F16 probe targeting the Igκ enhancers), and blue (nuclear lamin). *When a given cell is shown in two images, Igκ loci were found in different sections.

Our data revealed that E47 binds to the Igκ intronic/3´ enhancer region mostly monoallelically (Fig. 7a, b-d). We observed a substantial frequency of monoallelic association in pro-B cells, and the frequency of association increased following IL-7 withdrawal: by day 4 about 50% of cells showed monoallelic association of E47. Also, we observed a small fraction of cells (<10%) showing biallelic association (Fig. 7a, e-g) in all cases. However, in about 40-50% of cells at any given time point, there was no association between E47 and the Igκ enhancers observed. Thus, these findings demonstrate that E47 interacts with the Igκ enhancers mainly on one allele and

the frequency of such monoallelic association increases in pre-B cells.

E47 association correlates with more compact Igκ locus configuration and central subnuclear localization

Our previous results demonstrated the monoallelic association of E47 with the Igκ enhancers, and this suggests that such differential interaction might determine the choice of allele during immunoglobulin rearrangement. However, in order to assess if E47 indeed plays a role in allelic exclusion, it is necessary to show that E47 association somehow correlates with increased locus accessibility. Therefore, we selected the cells showing the monoallelic association of E47 with the Igκ enhancers, measured the spatial distances between the distal Vκ region and the Igκ enhancer region, and compared the distances between the E47-associated alleles and the non-associated alleles. Our analysis revealed that the average spatial distances between the distal Vκ region and Igκ enhancers were smaller on the E47-bound alleles compared to the non-bound alleles. However, in order to understand the statistical significance of such differences, a two-tailed t-test was performed pair-wise. Based on the *p* values, only the difference in the spatial distances on day 2 was significant (Tab. 2). While this alone cannot conclusively establish the correlation, based on the overall trend in the average spatial distances and the t-test data from day 2 post IL-7 withdrawal, we concluded that E47 association correlates with more compact locus configuration, perhaps making it a more favorable environment for rearrangement.

Table 2. Comparison of spatial distances between Vκ 2-135 and Eiκ/3´Eκ between E47-bound and unbound alleles in cells showing monoallelic association of E47 and the Igκ enhancers. (N = the number of cells in which E47 associates with the Igκ enhancers monoallelically.). *p* values were calculated in comparison to non-colocalizing alleles within each subgroup.

	Pro-B	Pre-B (Day 2)	Pre-B (Day 4)
N	22	30	43
colocalizing allele	0.31 ± 0.13 (ns)	0.28 ± 0.13 (*)	0.30 ± 0.12 (ns)
non-colocalizing allele	0.35 ± 0.11	0.35 ± 0.09	0.32 ± 0.15

Table 3. Comparison of subnuclear localization of E47-bound and unbound alleles in cells showing monoallelic association of E47 and the Igκ enhancers. Shown are average distances between the distal Vκ region or the Igκ enhancer region and nuclear membrane. *p* values were calculated in comparison to non-colocalizing alleles within each subgroup.

		Pro-B	Pre-B (Day 2)	Pre-B (Day 4)
N		22	30	43
Vκ 2-135	colocalizing allele	0.59 ± 0.22 (*)	0.63 ± 0.28 (ns)	0.54 ± 0.32 (ns)
	non-colocalizing allele	0.50 ± 0.23	0.55 ± 0.27	0.52 ± 0.26
Eiκ/3´Eκ	colocalizing allele	0.61 ± 0.30 (ns)	0.65 ± 0.27 (ns)	0.59 ± 0.27 (ns)
	non-colocalizing allele	0.54 ± 0.24	0.60 ± 0.22	0.60 ± 0.33

Based on the same idea, we wanted to see if E47 association might correlate with more central locus localization, another indicator of locus accessibility. The spatial distances between the distal Vκ region or Igκ enhancer region and nuclear membrane were compared between the E47-bound alleles and the non-bound alleles. In both pro-B and pre-B cells, we saw more central localization associated with E47 binding (Tab. 3), but this difference was statistically significant only based on the subnuclear localization of the distal Vκ region at the pro-B cell stage. On day 2 post IL-7 withdrawal, the E47-

bound alleles seemed farther away from nuclear periphery, but the difference was statistically non-significant based on the t-test. Interestingly, on day 4 following IL-7 withdrawal, we saw a reversed pattern of localization: although there was not much difference in the distances between the distal Vκ region and nuclear membrane, when we looked at the enhancer region, the E47-bound alleles actually were closer to nuclear periphery. However, based on the overall pattern, our data seem to indicate that E47 binding associates with more central locus localization.

Discussion

During B cell development, allelic exclusion ensures that the productive rearrangement of immunoglobulin heavy and light chain genes takes place at one of two given alleles, resulting in a monospecificity of all surface receptors. However, this process brings a unique challenge for selective rearrangement: given the ubiquitous nature of the recombination machinery and recombination signal sequences (RSSs), there arises a need for mechanisms that determine which immunoglobulin loci or allele undergo rearrangement. Although feedback inhibition is known to be an important mechanism for maintaining allelic exclusion, this idea alone is insufficient to account for the establishment of allelic exclusion[51]. Thus, it has been suggested that differential accessibility to the recombination machinery determines which allele would rearrange[2]. Furthermore, it has been proposed that differential accessibility, in turn, is established by the interaction between trans-acting factors and enhancers or other cis-acting regulatory sequences, resulting in a cellular environment conducive to the rearrangement of a given allele[17,41,42]. In this study, we present evidence suggesting that the differential association of E47 with the Igκ intronic/3´ enhancers might contribute to the establishment of allelic exclusion.

The Igκ locus is in a compact configuration in pro-B and pre-B cells

A broader implication of allelic exclusion is the choice of immunoglobulin locus for rearrangement at different stages during early B cell development. For instance, Ig gene rearrangement is sequentially controlled so that IgH genes undergo rearrangement in pro-B cells as opposed to in pre-B cells, despite the availability of the

recombination enzymes to the IgL loci. Therefore, it would be important to know how locus environments differ among different developmental stages within the same lineage or even different lineages. Therefore, we compared the spatial distances between the distal Vκ region and the Eiκ/3´Eκ enhancer region in pro-B, pre-B, and A12 cells, and it was shown that the Igκ loci are in a more compact state in both pro-B cells and pre-B cells, compared to A12 cells, which are non-B cells. This observation demonstrates that the lineage-specific control of locus environment may allow for the rearrangement of appropriate immunoglobulin genes at a given developmental stage. Interestingly, the Igκ locus was already compact in pro-B cells, and such compaction of the locus might explain why Igκ rearrangement could be observed before Ig heavy chain expression in pro-B cells[46].

In addition, we looked at the localization of the Igκ loci relative to nuclear periphery. As expected from our findings, the Igκ loci were more centrally positioned in pro-B and pre-B cells, reflecting their relatively open configuration. On the contrary, the Igκ loci remained close to nuclear periphery in A12 cells. Thus, along with locus compaction, the central localization of the Igκ loci reflects increased locus accessibility in pro-B and pre-B cells. We also observed that the Igκ enhancer region is closer to nuclear membrane in A12 cells than the distal Vκ region is, whereas both the distal Vk region and the enhancer region show comparable subnuclear positioning in pro-B and pre-B cells. We previously saw a similar pattern with the IgH enhancer region and its differential nuclear localization in pro-B and A12 cells (data not shown). This raises the possibility that the subnuclear positioning of such regulatory elements within the Ig loci may have a role in promotion of locus accessibility, but this is yet to be proven.

E47 associates with the Igκ intronic/3´ enhancers predominantly monoallelically

In order to understand the nature of association between E47 and the Igκ enhancers, we utilized the novel combination of the ReAsH-TC labeling system and 3D FISH and noted the frequency of monoallelic, biallelic, and no colocalization of E47 with the Igκ enhancers in pro-B and in vitro differentiated pre-B cells. This study was the first to demonstrate that E47 associates with the Igκ enhancers monoallelically in pre-B cells. Also it should be noted that we observed a significant incidence of monoallelic association in pro-B cells. However, this was consistent with the previous finding that E boxes in both Eiκ and 3´Ek enhancers are already occupied in pro-B cells[61]. Also this might explain why the Igκ loci are in a compact configuration in pro-B cells as we observed previously.

Since the notion of differential locus activation in allelic exclusion would be most easily explained by the monoallelic binding of E47, this finding confirmed our initial hypothesis. However, we also observed a non-negligible proportion of pro-B and pre-B cells showing the biallelic association of E47 and the Igκ enhancers. However, this observation does not necessarily contradict the idea of differential locus accessibility in allelic exclusion. The existing model of allelic exclusion allows for the possibility that the second allele can become accessible and undergo rearrangement if the rearrangement of the first allele is not productive[17]. Furthermore, even following the productive arrangement on the first allele, trans-acting factors can be still present[17] and potentially interact with regulatory elements on the second allele. However, since RAG expression is down-regulated upon the expression of a functional, productive surface receptor, the second allele can be in an open configuration, yet do not undergo

rearrangement[17]. Nevertheless, it has been known that the expression of a functional surface receptor also reduces the expression of E2A via tonic signaling[43]. Thus, tonic signaling should reduce the incidence of biallelic association. However, because E47 is over-expressed in this study, and since the level of E2A has been shown to be the rate-limiting step in the Igκ gene rearrangement as demonstrated in E2A heterozygous mice[50], a high level of E47 under the over-expression vector system may contribute to biallelic association. Thus, it is possible that such biallelic association may not be observed at the endogenous expression level of E2A.

Monoallelic E47 association correlates with differential locus compaction and subnuclear localization

The monoallelic association of E47 has an important implication in the mechanism of allelic exclusion, but it alone is not sufficient to explain how allelic exclusion is achieved. Based on the fact that trans-acting factors can mediate chromatin changes, it would be imperative to draw a connection between E47 binding and more open locus configuration. In this study, we show that the Igκ locus is more compact on the E47-bound alleles than the unbound alleles. A statistically significant difference was observed on day 2 post IL-7 withdrawal only, and the same trend was observed on day 0 and 4. The lack of statistical significance at some of the time points might be attributed to the over-expression of E47, which can potentially activate its downstream targets and affect locus accessibility indirectly. Therefore, repeating this study using the endogenous expression level of E47 would be insightful. Also, human E47 cDNA was used for this study, and it is not clear how this would affect locus compaction

differently than mouse E47. However, based on the overall trend of the spatial distances, E47 binding seems to mediate locus compaction.

We also studied the subnuclear localization of the Igκ locus as an alternative strategy to see if E47 association correlates with an open locus environment. We observed a correlation between nuclear localization and E47 association in pro-B cells showing the monoallelic association. However, such correlation was not supported by the *p* value in pre-B cells on day 2, and on day 4 both E47-bound and unbound alleles showed similar subnuclear localization with respect to the distal Vκ region, and the Eiκ/3´Eκ region was in fact closer to nuclear periphery on the E47-colocalizing alleles. This observation was in contrast to the previous finding that only one Igκ allele localizes to nuclear periphery in pre-B cells[17,18]. Such inconsistencies might stem from the secondary effect of E47 over-expression or a relatively low sample number used for statistical analysis. Furthermore, since the statistical analyses were done on the differentiating cells, it is the mixed populations of $CD25^+$ and $CD25^-$ cells that might have led to such statistical inconsistencies. However, based on the overall trend, it seems that there is a correlation between E47 association and more central subnuclear localization at the pro-B and early pre-B cell stages.

Potential mechanisms of how E47 might regulate locus accessibility

Our findings demonstrate that the nature of E47 association can control locus accessibility. The next logical question to ask would be how E47 can mediate this. One possible mechanism is histone acetylation, which has been implicated in chromatin remodeling. Interestingly, E2A proteins are known to have potential to control histone

acetylation. It has been shown that E2A-deficient pre-B cells show impaired histone acetylation at Igκ intronic and 3´ enhancers and reconstitution with E47 in these cells can promote Igκ histone acetylation[29]. Furthermore, E2A has been shown to associate with histone acetylatransferases such as p300 and CBP[7,36]. Along with the previous observation that enhancers can recruit histone modifying enzymes to IgH loci[37], these previous findings suggest that E2A may be directly involved in histone acetylation, and E47 might act as a bridge between allelic exclusion and histone acetylation. Thus, future studies might include the simultaneous visualization of E47 with the Igκ enhancers and histone modifying enzymes at the Igk locus. If the monoallelic association of E47 and histone modifying enzymes is shown to promote differential locus accessibility with the staining techniques we used, this would further support our findings.

However, it is unlikely that E2A alone plays a role in Igκ activation. For instance, the ectopic expression of early B cell factor (EBF) along with RAG proteins can induce IgH and IgL gene rearrangement in non-lymphoid cells[16,55]. Therefore, E2A probably promotes locus accessibility along with other factors. Especially in the context of how E2A can promote locus accessibility, one possible candidate is IRF-4. Interferon regulatory factor 4 (IRF-4) and related gene family member IRF-8 are known to be necessary for the pro-B to pre-B cell transition and Igk locus activation[33]. It has been shown that especially IRF-4 can directly bind 3´Eκ and required for Igκ activation in pre-B cells[33]. Previous studies have shown that IRF-4 can interact with E2A at the 3´Eκ cooperatively and IRF-4 promotes the recruitment of E2A to 3´Eκ[45]. Furthermore, IRF-4 deficiency leads to significant reduction of histone acetylation at both Igκ enhancers[29].

These findings suggest that E2A and other factors have potential to activate Ig loci through histone modification and other mechanisms. Since our study implies that E2A is at least partly responsible for differential locus accessibility, it would be important to find out what other factors can promote Iκ locus activation along with E2A.

While the monoallelic nature of the interaction between trans-acting factors and cis-acting enhancers could be one of several mechanisms for the establishment of allelic exclusion, additional studies will be necessary to truly understand the implication of our findings. First of all, it would be important to study this phenomenon at the endogenous expression level of E47. This could be done by creating knock-in mice with a tetracysteine-tagged E2A exon in the endogenous locus and visualizing the association of E47 with the Igκ locus in primary pro-B and pre-B cells. If our findings were to be replicated using such transgenic mice, this would further support the validity of our results. Furthermore, it would be worth seeing if E12 could be involved in locus activation as its role in immunoglobulin gene rearrangement is largely unknown. Furthermore, Igκ intronic and 3´ enhancers are known to have differential functions in the context of Igκ rearrangement and gene expression[5,31]. Because this study uses a BAC probe spanning both intronic and 3´ enhancers, it does not differentiate between intronic enhancer and 3' enhancer. Thus, it would be important to pinpoint which enhancer E47 binds, and this can be done by using smaller DNA probes. This might reveal differential or synergistic roles of both enhancers in Igκ locus activation prior to rearrangement.

In this study, we have shown that E47 associates with the Igκ enhancers monoallelically in pro-B and pre-B cells, and that the E47-associated alleles correlate

with more open locus compared to the non-associated alleles. This study represents a potential control mechanism for the establishment of allelic exclusion at the Igκ loci. Furthermore, the implication of monoallelic interaction between trans-acting factors such as E2A and regulatory DNA elements might be extended to allelic exclusion observed at IgH and TCR loci.

Materials and Methods

Materials

ReAsH was a gift from Ben Giepmans in the Mark Ellisman Laboratory (University of California, San Diego, La Jolla, CA).

Mice and cell culture

All mice used were 6- to 8-week-old WT mice maintained on the C57BL/6 background. Pro-B cells were generated from femoral and tibial bone marrow suspensions by purification of $B220^+$ cells using magnetic separation (Miltenyi Biotec) and in vitro expansion for 7-8 days in Opti-MEM medium containing 10% fetal calf serum (FCS), 2% penicillin/streptomycin/L-glutamine (PSG), and 50μM β-mercaptoethanol (β-ME) supplemented with IL-7 and SCF.

Generation of MinVTAC E47TC construct

pCSretTAC-E47TC was previously obtained from Kristina Beck. Both pCSretTAC-E47TC and MinVTAC E47 were digested with the restriction enzyme XhoI. The restriction fragment containing the part of E47 cDNA and tetracysteine coding sequence from pCSretTAC-E47TC was ligated into MinVTAC E47 using T4 DNA ligase (Invitrogen). The tetracysteine motif FLNCCPGCCMEP encoded by MinVTAC E47TC was previously optimized for the increased affinity for ReAsH and dithiol resistance[35].

Transfection for retrovirus production

HEK293T cells were used for retrovirus production. They were cultured in DMEM with 10% FCS + 2% PSG. The medium was refreshed prior to transfection. For a single transfection, 21 µg of either MinV Bcl-2 or MinVTAC E47TC and 9 µg of pCL plasmid were added to deionized water up to 438 µl. Then 62 µl of 2M $CaCl_2$ were added to the DNA-dH_2O mixture. The 500µl suspension was added dropwise to 500µl 2x HBS while vortexing the tube. Subsequently 1ml of the mixture was added to culture medium dropwise. The medium was aspirated 8h following transfection, and HEK293T cells were incubated in Opti-MEM containing 10% FACS, 2% PSG, and 50µM β-ME. Viral supernatants were collected 24h and 48h following transfection.

Retroviral transduction and IL-7 withdrawal

After 7-8 days in culture, pro-B cells were transduced with MinV Bcl-2 construct. The viral supernantant supplemented with hexadimethrine bromide at 4µg/ml was incubated on ice for 10min. Cells were resuspended in viral supernantant at the final concentration of 1 x 10^6 cells/ml, and the cell-virus suspensions were transferred to a 6-well plate. Cells were centrifuged for 1.5h at 2500 rpm at 30°C. Following infection, cells were incubated for 2h at 37°C. Subsequently cells were removed from the 6-well plate and resuspended in Opti-MEM medium supplemented with IL-7 and SCF at the concentration of 1.5 x 10^6 cells/ml. The following day, cells were transduced with MinVTAC E47TC construct. Following the second infection, cells were incubated for two days, and huCD25$^+$ cells were purified using magnetic seperation (Miltenyi Biotec). In order to remove IL-7, purified huCD25$^+$ cells were washed three times with

Opti-MEM lacking IL-7 and SCF and incubated in the same medium at 37°C. The medium was refreshed every other day following IL-7 withdrawal.

Flow cytometry

Fc receptors were blocked with anti-mouse CD16/32 antibody (eBioscience) for 20 min on ice. Cells were stained with appropriate antibodies diluted in 0.1 ml FACS buffer (1x PBS/1% FCS) for 20 min on ice. Cells stained with biotinylated antibodies were washed once with 1 ml of FACS buffer and incubated with streptavidin-conjugated allophycocyanin (eBioscience) for 20 min on ice. The following antibodies were used for surface staining and purchased from either eBioscience or BD Biosciences: peridinin chlorophyll protein-conjugated anti-mouse CD45R/B220 (RA3-6B2), fluorescein isothiocyanate-conjugated anti-mouse CD43 (eBioR2/60), phycoerythrin-conjugated anti-mouse CD25 (PC61), and biotinylated anti-mouse IgM (11/41). Following surface staining, cells were analyzed on FACScalibur^{TM} flow cytometer (Becton Dickinson).

ReAsH labeling

Prior to labeling, cells were washed twice with HBSS. For each staining, ReAsH labeling solution was freshly prepared by incubating 1mM ReAsH/10mM 1,2-ethanedithiol (EDT) in DMSO for 10 min at room temperature. The dye mixture was then diluted 1:1000 in Hank's balanced salt solution (HBSS; Invitrogen). Cells were suspended in 1ml of ReAsH labeling solution at 1.5 x 10^6 cells/ml. Cells were incubated for 1.5 h at 37°C. After labeling, cells were rinsed once with HBSS, followed by three

separate 10-min incubations with 100 µM EDT in HBSS at 37°C. Subsequently cells were rinsed twice with HBSS to remove residual EDT. Stained cells were applied onto coverslips treated with poly-L-lysine (Invitrogen) and incubated for 10 min at 37°C. The coverslips were washed once with 1x PBS to remove unattached cells, and subsequently the cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT). Coverslips were washed once with 1x PBS and treated with 0.1M Tris-Cl (pH 7.2) for 10 min at RT. Then cells were washed once in 1x PBS, and were either used immediately for combined immunoflourescence/3D FISH or stored in 0.1% sodium azide in 1x PBS at 4°C for up to 1 month.

Combined immunofluorescence and 3D FISH

Following ReAsH staining and fixation, cells were permeabilized with 1x PBS/0.1% Triton X-100/0.1% saponin for 10 min at RT, and subsequently incubated with 20% glycerol/1x PBS solution for 20 min at RT. Then coverslips were immersed in liquid nitrogen and thawed at RT, and two additional freeze-thaw cycles were done. Coverslips were washed once with 1x PBS and incubated in 1x PBS/0.1% Triton X-100/5% bovine serum albumin (BSA) for 30 min at 37°C. The nuclear membrane was stained with anti-lamin A and B antibodies (Santa Cruz) diluted in 1x PBS/0.1% Triton X-100/5% BSA for 30 min at 37°C. Coverslips were washed twice in 1x PBS/0.1% Triton X-100/0.1% saponin for 10 min at RT with gentle agitation. Subsequently cells were incubated with biotinylated anti-goat donkey antibodies supplemented with 5% normal donkey serum for 30 min at 37°C. Coverslips were washed twice in 1x PBS/0.1% Triton X-100/0.1% saponin for 10 min at RT. Then cells were washed once

with 1x PBS and fixed with 2% paraformaldehyde in 1x PBS for 10 min at RT. Coverslips were incubated in 0.1 M Tris-Cl (pH 7.2) for 5 min at RT, and washed once with 1x PBS. Subsequently cells were treated with 0.1 M HCl for 30 min at RT, washed once with 1x PBS, and incubated in 1x PBS/3% BSA solution supplemented with RNase A for 1h at 37°C. Then coverslips were washed with 1x PBS, and treated with 1x PBS/0.5% Triton X-100/0.5% saponin for 30 min at RT. Coverslips were washed once with 1x PBS, and incubated in 2x SSC/70% formamide solution (pH. 7) for 2.5 min at 73°C, followed by an 1-min incubation in 2x SSC/50% formamide solution (pH. 7) at 73°C. Then excess formamide on coverslips was removed and 10 µl of hybridization cocktail was added to each coverslip. Coverslips were mounted on glass slides, sealed with rubber cement, and incubated overnight at 37°C. The hybridization cocktail contained 400 ng of each labeled probe, 16 µg of mouse Cot-1 DNA, and 2 µg of salmon sperm DNA in 50% formamide, 2x SSC, 10% dextran sulfate. The probes were denatured for 5 min at 75°C, and chilled on ice. RP24-279F16 were labeled using a nick translation kit (Roche) with Alexa-fluor 488-5-dUTP (Molecular Probes). BAC RP23-234A12 was labeled using a digoxigenin (DIG) nick translation kit (Roche). Following 37°C overnight incubation, coverslips were removed from glass slides, and washed once in 2x SSC/50% formamide (pH. 7) for 15 min with gentle agitation, and three times in 2x SSC 5 min each at 37°C with gentle agitation. Subsequently coverslips were incubated in 2x SSC/3% BSA/0.1% Tween-20 for 30 min at RT. The DIG-labeled BAC probe and lamin antibodies were detected by incubating cells with Cy5-conjugated mouse anti-DIG antibodies (Jackson Immunoresearch) and Marina Blue-conjugated neutravidin (Molecular Probes) diluted in 2x SSC/3% BSA/0.1% Tween-20 for 30 min

at 37°C. Then coverslips were washed twice with 2x SSC/0.1% Tween-20 for 10 min each at RT with gentle agitation. Coverslips were washed once with 1x PBS at RT and mounted on glass slides with Prolong.

Image acquisition and spatial distance calculation

Images were acquired with the DeltaVision deconvolution microscope system (Applied Precision, Inc.) located at the UCSD Cancer Center microscope facility. Using a 100x (NA 1.4) lens, the images of 30-40 serial optical sections, spaced by 0.2 µm, were acquired. The data sets were deconvolved and optical sections superimposed to produce 3D images using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation. The 3D coordinates of the center of mass of each probe were input into Microsoft Excel, and the distances separating each probe were calculated using the equation

$$\sqrt{(X_a - X_b)^2 + (Y_a - Y_b)^2 + (Z_a - Z_b)^2}$$

where X, Y, Z are the coordinated of object a or b. *p* values were calculated with ANOVA test using nonparametric methods. All statistical tests were done on GraphPad Prism version 4.0.

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