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Association between the *Igk* and *Igh* immunoglobulin loci mediated by the 3′ *Igk* enhancer induces 'decontraction' of the *Igh* locus in pre–B cells

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Variable-(diversity)-joining (V(D)J) recombination at loci encoding the immunoglobulin heavy chain (*Igh*) and immunoglobulin light chain (*Igk*) takes place sequentially during successive stages in B cell development. Using three-dimensional DNA fluorescence *in situ* hybridization, here we identify a lineage-specific and stage-specific interchromosomal association between these two loci that marks the transition between *Igh* and *Igk* recombination. Colocalization occurred between pericentromerically located alleles in pre–B cells and was mediated by the 3′ *Igk* enhancer. Deletion of this regulatory element prevented association of the *Igh* and *Igk* loci, inhibited pericentromeric recruitment and locus 'decontraction' of an *Igh* allele, and resulted in greater distal rearrangement of the gene encoding the variable heavy-chain region. Our data indicate involvement of the *Igk* locus and its 3′ enhancer in directing the *Igh* locus to a repressive nuclear subcompartment and inducing the *Igh* locus to decontract.

B cells and T cells express lineage-specific antigen receptors that mediate humoral and cellular immunity, respectively. Immunity depends on the regulated recombination of variable (V), diversity (D) and joining (J) gene segments at loci encoding B cell and T cell antigen receptors. This generates sufficient receptor diversity to enable the recognition of a nearly limitless array of potential antigens. Each antigen receptor consists of heavy and light chains, each encoded by distinct loci. Because common factors are required for V(D)J recombination at all immune receptor loci, developmentally regulated changes in locus accessibility are crucial for regulating this process[1].

Regulation of accessibility is exerted at many levels to ensure lineage specificity and sequential rearrangement of gene segments in loci encoding the immunoglobulin heavy chain (*Igh*) and immunoglobulin light chain (*Igk*)[2]. Tight regulation is also essential for protecting the genome from such rearrangement events, which require the generation of inherently unstable and potentially dangerous double-stranded DNA breaks. Allelic exclusion, the process by which the successful production of one immunoglobulin chain suppresses further rearrangement at the other allele of the same immunoglobulin locus, represents an integral part of this regulation and ensures clonality and monospecific recognition by the B cell antigen receptor on individual lymphocytes, which is a basic tenet of the acquired immune response.

Recombination starts at the pro–B cell stage with D-J rearrangement of both *Igh* alleles. Synapse formation of gene segments separated by a large distance is facilitated by looping, which results in locus contraction in cells undergoing rearrangement[3,4]. Functional V-D-J rearrangement at one *Igh* allele leads to expression of immunoglobulin μ-chain as part of the pre–B cell antigen receptor. Signaling through this receptor enforces cessation of further *Igh* rearrangement and triggers a burst of proliferation of large pre–B cells, which subsequently differentiate into small pre–B cells in which *Igk* rearrangement takes place[5]. Locus contraction mediated by looping occurs before the onset of *Igk* germline transcription[6]. *Igk* rearrangement occurs after the onset of transcription of the unrearranged cluster of J gene segments and depends on well-characterized enhancers located in the joining-constant (J-C)–region intron and matrix-attachment region (MiEκ) and 3′ of the C-region exon (3′Eκ). Deletion of the two enhancers, individually or simultaneously, diminishes or abrogates V-J *Igk* rearrangement, respectively[7–9].

Allelic exclusion at the *Igh* locus, established at the pre–B cell stage of development by changes in chromatin accessibility[10], is thought to be crucial for preventing ongoing rearrangement of the second partially assembled (DJ-rearranged) *Igh* allele when the V(D)J recombinase is re-expressed for the purpose of *Igk* rearrangement. Accumulating evidence supports a 'feedback inhibition' model of establishing allelic exclusion of the *Igh* locus, but the detailed molecular basis of

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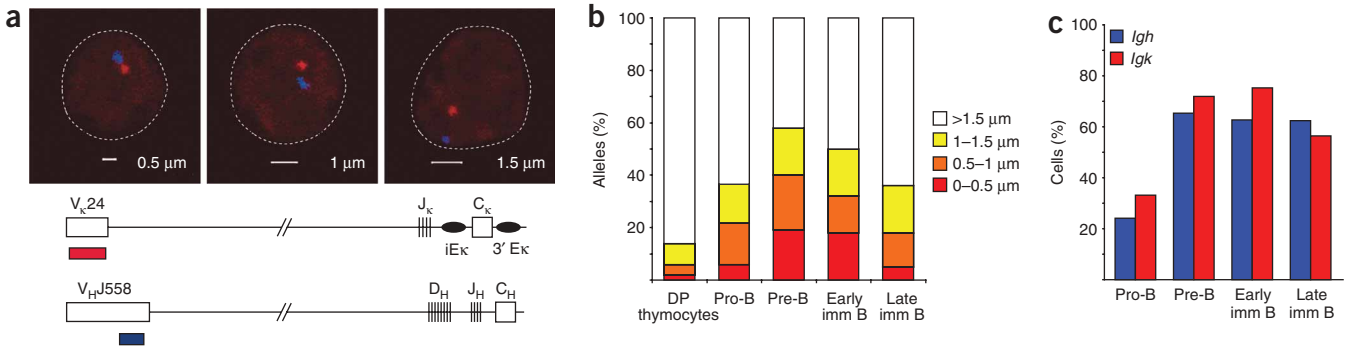

Figure 1 Interchromosomal association between one *Igh* allele and one *Igk* allele coincides with pericentromeric recruitment of the two loci. (**a**) Confocal microscopy of the distances separating the 5′ ends of the *Igh* and *Igk* alleles (top row); scale bars, 0.5 μm (left), 1.0 μm (middle) and 1.5 μm (right). Below, positions of probes used for detecting *Igh* (CT7-526A21) and *Igk* (RP23-11G13). (**b**) Percent alleles with various distances (key) separating the 5′ ends of the *Igh* and *Igk* loci in subsets of sorted bone marrow cells and thymocytes (percentage and sample sizes, **Supplementary Table 1**). Cells with no close association between *Igh* and *Igk* alleles (over 1.5 μm) are considered as having separation of a single pair of alleles. (**c**) Three-color three-dimensional DNA FISH analysis of the pericentromeric location of *Igh* and *Igk* alleles during B cell development, with the *Igk* probes RP23-101G13 (V_κ24) and RP24-387E13 (*Igk* C region) plus a γ-satellite probe, and (separately) with the *Igh* probes CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) plus a γ-satellite probe. Values indicate percent cells with monoallelic association of *Igh* and *Igk* with pericentromeric clusters (percentages and sample sizes, **Supplementary Table 2**). DP, double positive; imm, immature. Data are representative of three experiments.

this model is yet to be defined[2]. However, pericentromeric recruitment is known to be involved in establishing and maintaining allelic exclusion of all immunoglobulin loci[4,11,12]. After successful recombination of one *Igh* allele, repositioning of the second allele to pericentromeric heterochromatin (a repressive compartment of the nucleus) decreases accessibility of *Igh* to the recombinase during *Igk* rearrangement[4]. In contrast, repositioning of the *Igk* allele to pericentromeric clusters occurs at the pre–B cell stage, before the onset of *Igk* rearrangement, and may limit accessibility of the *Igk* locus to recombinase to a single (euchromatic) allele[12]. In addition, 'decontraction' of the *Igh* locus occurs at the same developmental stage. This process contributes to allelic exclusion by physically separating distal and middle variable heavy-chain (V_H) gene segments from the proximal D-J domain of the locus, thereby preventing further synapse formation and ongoing rearrangement between these regions[4]. Recruitment of the not-yet-rearranged *Igk* allele and the partially rearranged *Igh* allele to pericentromeric heterochromatin and decontraction of the partially rearranged *Igh* allele occur at the same developmental stage, which suggests the existence of a coordinated event. That prompted us to examine the locations of these two loci relative to each other and to further investigate the factors required for changes in conformation that occur at the *Igh* locus during B cell development.

RESULTS

Interchromosomal association between immunoglobulin loci

To examine the positions of the *Igh* and *Igk* loci, we used two-color three-dimensional DNA fluorescence *in situ* hybridization (FISH) with DNA probes generated from two bacterial artificial chromosomes, CT7-526A21 and RP23-101G13; these probes map to the 5′ end of the *Igh* locus on chromosome 12 and the 5′ end of the *Igk* locus on chromosome 6, respectively. In each cell, either the two alleles of both loci were well separated or one *Igh* and one *Igk* allele

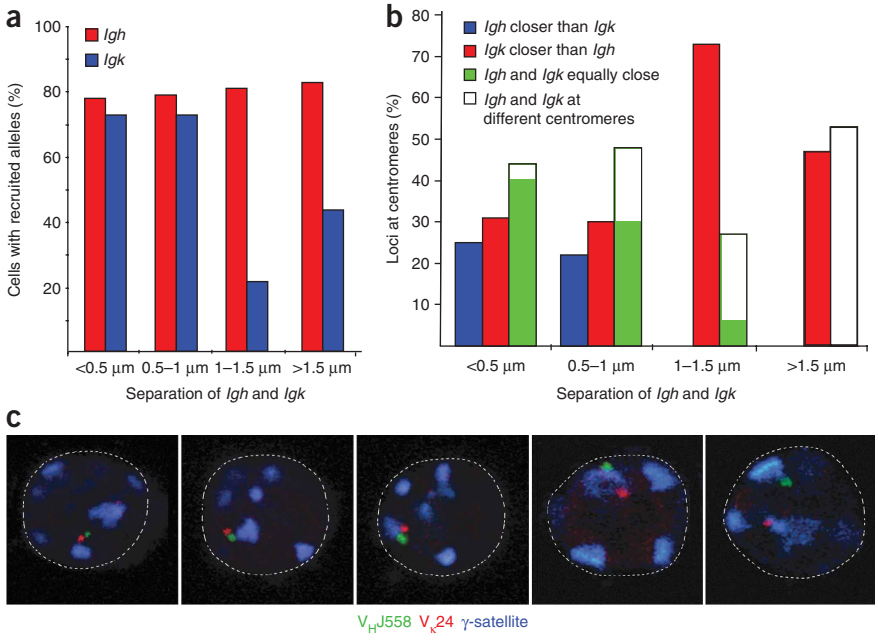

Figure 2 The association between *Igh* and *Igk* occurs on the pericentromerically recruited allele. (**a**) Three-color three-dimensional DNA FISH analysis of pre–B cells with the *Igk* and *Igh* probes described in **Fig. 1a**. Values indicate localization of *Igh* and *Igk* alleles at various distances apart (horizontal axis), relative to pericentromeric heterochromatin (percentage and sample sizes, **Supplementary Table 3**). (**b**) Evaluation of the orientation of *Igh* and *Igk* alleles at various distances apart, relative to pericentromeric heterochromatin (percentage and sample sizes, **Supplementary Table 4**). (**c**) Confocal microscopy showing various facets of interacting loci relative to pericentromeric heterochromatin. Original magnification, ×100. Data are representative of three experiments.

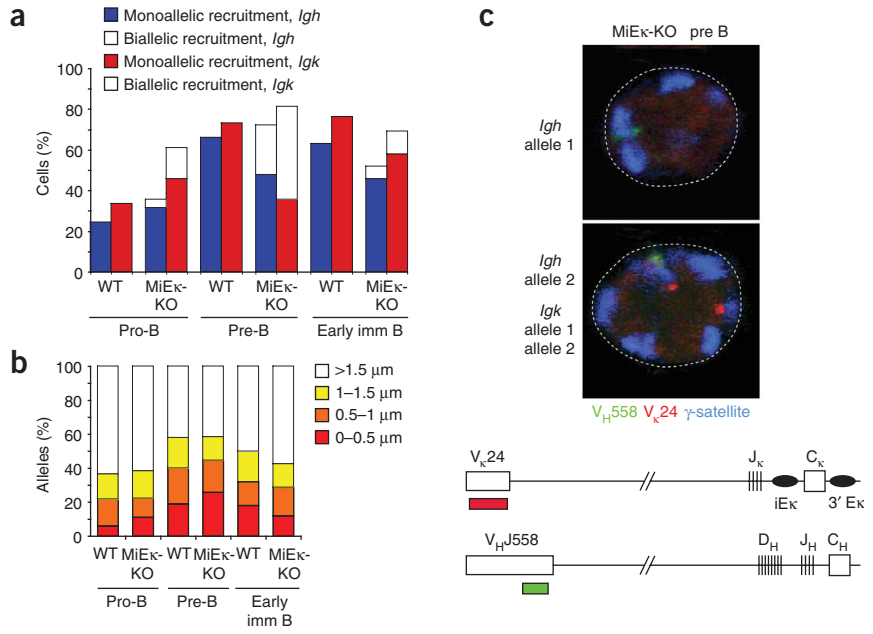

Figure 3 Increased pericentromeric recruitment of *Igk* promotes a higher frequency of interchromosomal association between *Igh* and *Igk* and more pericentromeric recruitment of *Igh* dependent on 3′Eκ. (**a**) Three-color three-dimensional DNA FISH of the pericentromeric location of *Igh* and *Igk* alleles (as described in **Fig. 1**) in cells sorted from wild-type (WT) and MiEκ-knockout (MiEκ-KO) bone marrow at various developmental stages (percentages and sample sizes, **Supplementary Table 2**). (**b**) Distance separating the 5′ ends of the *Igh* and *Igk* loci in pro–B cell, pre–B cell and early immature B cell (Early imm. B) subsets of sorted bone marrow cells from wild-type and MiEκ-knockout mice (percentage and sample sizes, **Supplementary Table 1**). Data are not shown for the late immature B cell subset, as the mutant mice do not have these cells. (**c**) Top, confocal microscopy of biallelically recruited *Igh* and *Igk* in pre–B cells from MiEκ-knockout mice. Original magnification, ×100. Below, positions of probes used for detecting *Igh* and *Igk*. Data are representative of three experiments.

were present in close spatial proximity. We grouped measurements of the distance separating the two loci into the following four categories: less than 0.5 μm apart, 0.5–1 μm apart, 1–1.5 μm apart and over 1.5 μm apart (**Fig. 1a**). Our measurements related to the distance separating the pair of alleles localized together in each cell. When we found no close association between *Igh* and *Igk* alleles in an individual cell (over 1.5 μm apart), we considered this to be separation of a single pair of alleles. We also used probes mapping to the *Igh* and *Igk* C regions to rule out the possibility that deletion of distal V_H gene regions affected observations of the frequency of association of the two loci (**Supplementary Fig. 1** online). Using various subsets of B cells sorted from mouse bone marrow, we examined the positions of the two loci relative to that of pericentromeric heterochromatin, as described before[4]. Interchromosomal association occurred at low frequency in pro–B cells, but it increased and peaked during the pre–B cell stage, as shown by the substantially greater frequency of loci separated by less than 0.5 μm, and it decreased during the late immature B cell stage (**Fig. 1b** and **Supplementary Table 1** online). Colocalization was lineage specific, as we noted no substantial *Igh-Igk* pairing in $CD4^+CD8^+$ thymocytes (**Fig. 1b**). In addition, the *Igh-Igk* association coincided temporally with pericentromeric recruitment of *Igh* and *Igk* (**Fig. 1c** and **Supplementary Table 2** online).

Association on pericentromerically recruited alleles

Colocalization of *Igh* and *Igk* occurred on one pair of alleles in each cell at a stage in development during which one allele from each locus is often repositioned at pericentromeric heterochromatin. Thus, we sought to determine which alleles participated in the interchromosomal association. To address this, we did three-color three-dimensional DNA FISH of sorted pre–B cells with *Igh* and *Igk* probes, plus a γ-satellite probe mapping to pericentromeric heterochromatin. The most closely associated *Igh* and *Igk* alleles were positioned in heterochromatic regions (**Fig. 2** and **Supplementary Table 3** online). Where paired recruited alleles were separated by over 1 μm, the *Igk* allele frequently localized together with pericentromeric heterochromatin and was considered to be recruited to heterochromatic regions, whereas the *Igh* allele was positioned at a distance from the pericentromeric cluster and was not considered to be recruited to heterochromatic regions (**Fig. 2a** and **Supplementary Table 3**). This suggested that the two immunoglobulin loci do not simultaneously move to pericentromeric clusters but instead that recruitment of *Igk* might precede that of *Igh*. To analyze that hypothesis in greater detail, we compared the distance between the two loci with the orientation of *Igh* and *Igk* alleles in pericentromeric regions. When the two loci were separated by less than 1 μm, either *Igh* or *Igk* was positioned closer to pericentromeric heterochromatin, but more often they were both equally close to the same pericentromeric cluster or equally close to a different pericentromeric cluster (**Fig. 2b,c** and **Supplementary Table 4** online). Where immunoglobulin loci were separated by over 1.5 μm, all cells had either one *Igk* allele alone at pericentromeric heterochromatin or both *Igh* and *Igk* at different pericentromeric clusters. *Igh* was positioned at heterochromatic regions only when it associated with *Igk* at the same pericentromeric cluster or when both immunoglobulin loci were recruited to different pericentromeric clusters. *Igh* was never present at heterochromatic regions in pre–B cells in the absence of *Igk* recruitment. These observations suggested that pericentromeric recruitment of *Igk* was the initial event. The frequency of association was lower at the immature B cell stage of development, when *Igh* and *Igk* were positioned mainly at different pericentromeric clusters (**Fig. 1b,c**). These findings suggest that association between the two loci takes place at a shared pericentromeric cluster after recruitment of one *Igk* allele and that the two loci subsequently move apart to reposition at different pericentromeric clusters. Thus, pericentromeric recruitment of *Igk* may promote pericentromeric recruitment of *Igh*, perhaps through a transient association between the two loci. Therefore, although immunoglobulin alleles may be constrained to a repressive subcompartment of the nucleus, they do not necessarily remain static and can move from one pericentromeric cluster to another.

Sequential recruitment to pericentromeric regions

To further test the idea that the pericentromeric *Igk* allele is involved in the recruitment of an *Igh* allele to pericentromeric heterochromatin, we examined nuclear positioning in mice with targeted deletion of MiEκ or 3′Eκ. Published analysis of pre–B cells from these mice has

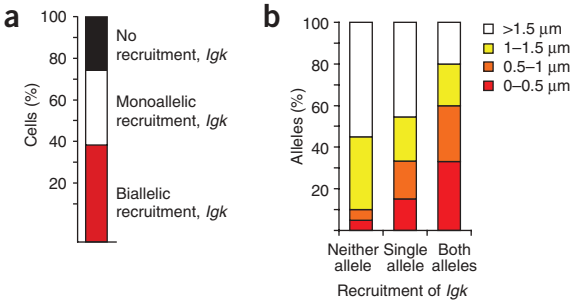

Figure 4 Association of *Igh* and *Igk* increases with increasing recruitment of *Igk* to pericentromeric regions. (**a**) Pre–B cells with monoallelically and biallelically pericentromeric *Igk* alleles and cells in which neither *Igk* allele is pericentromeric, in MiEκ-knockout mice. The cells analyzed here are from mice different from those in **Figure 3**. (**b**) Association of *Igh* and *Igk* in cells with monoallelically and biallelically pericentromeric *Igk* alleles and in cells in which neither *Igk* allele is pericentromeric (percentage and sample sizes, **Supplementary Table 5**). Data are representative of three experiments.

shown they have much less V_κ and J_κ germline transcription and V_κ-J_κ recombination[7,9]. Our three-dimensional DNA FISH analysis of sorted developing B cell subsets from MiEκ-knockout mice showed that pericentromeric recruitment of *Igk* occurred prematurely at the pro–B cell stage of development and that at the subsequent pre–B cell stage, nearly half the cells had an atypical appearance, with both *Igk* alleles located at pericentromeric heterochromatin (**Fig. 3a**). These results supported published data showing that absence of MiEκ leads to silencing of *Igk* transcription[9]. Notably, we found no defect in *Igk* locus contraction in MiEκ-knockout pre–B cells (**Supplementary Table 5** online). As a test of our model, we determined whether greater pericentromeric recruitment of *Igk* alleles in these mutants led to more localization together with *Igh* and a higher frequency of recruitment of *Igh* to heterochromatin. Pre–B cells from MiEκ-knockout mice had slightly more close association (less than 0.5 μm) between the *Igh* and *Igk* loci (**Fig. 3b** and **Supplementary Table 1**), and this correlated with the unusual appearance of a percentage of pre–B cells in which both *Igh* alleles were repositioned at pericentromeric heterochromatin (**Fig. 3a** and **Supplementary Table 2**). These data further suggested that recruitment of *Igk* influenced the recruitment of *Igh* to pericentromeric heterochromatin. To verify that conclusion, we examined the cells in which both *Igh* alleles were repositioned at pericentromeric clusters to determine whether biallelic recruitment of *Igh* was facilitated by biallelic recruitment of *Igk*. We used three-color three-dimensional DNA FISH to examine the positions of the two loci relative to that of pericentromeric heterochromatin. Analysis of the small number of cells in which both *Igh* alleles were located at pericentromeric heterochromatin showed that in 20 of 24 cells, both *Igh* and *Igk* were biallelically recruited to pericentromeric heterochromatin (**Fig. 3c**).

As the observed greater frequency of closely associating (0.5 μm) alleles in MiEκ-knockout pre–B cells was subtle, to definitively assess the affect of pericentromeric recruitment of *Igk* on the association between the two loci, we separately analyzed the frequency of association between *Igh* and *Igk* loci in the following populations from MiEκ-knockout pre–B cells: cells in which neither *Igk* allele was recruited; cells in which a single *Igk* allele was recruited; and cells in which *Igk* was biallelically recruited (**Fig. 4a**). *Igh*-*Igk* loci association was greater with more recruitment of *Igk* to pericentromeric heterochromatin, and we detected negligible close association in the absence of *Igk* localization in pericentromeric regions (**Fig. 4b** and **Supplementary Table 6** online). These data collectively indicate that pericentromeric localization of *Igk* promotes *Igk*-*Igh* association and the recruitment of *Igh* to pericentromeric heterochromatin.

The *Igh*-*Igk* close association requires 3′Eκ

Our analysis of allelic association and pericentromeric recruitment of *Igh* in 3′Eκ-knockout progenitor B cells provided a very different result from those reported above. *Igk* loci in these cells acted like the *Igk* loci in MiEκ-knockout developing B cells, in both cell types, *Igk* was prematurely repositioned to pericentromeric clusters at the pro–B cell stage of development, and at this and later stages, many cells had

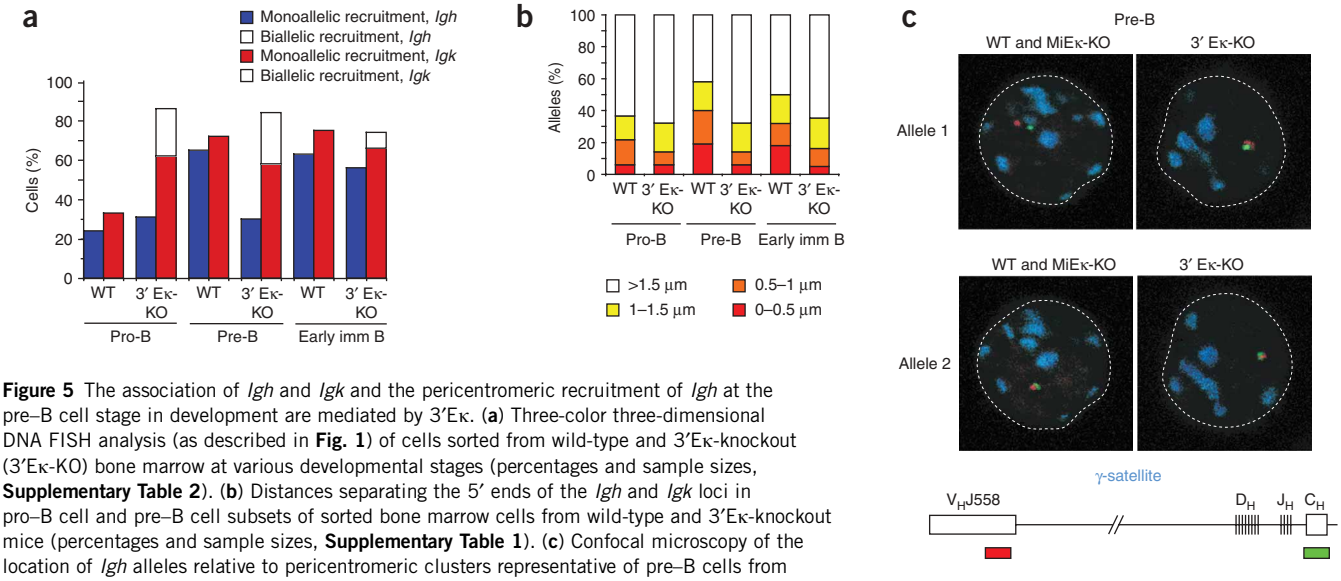

Figure 5 The association of *Igh* and *Igk* and the pericentromeric recruitment of *Igh* at the pre–B cell stage in development are mediated by 3′Eκ. (**a**) Three-color three-dimensional DNA FISH analysis (as described in **Fig. 1**) of cells sorted from wild-type and 3′Eκ-knockout (3′Eκ-KO) bone marrow at various developmental stages (percentages and sample sizes, **Supplementary Table 2**). (**b**) Distances separating the 5′ ends of the *Igh* and *Igk* loci in pro–B cell and pre–B cell subsets of sorted bone marrow cells from wild-type and 3′Eκ-knockout mice (percentages and sample sizes, **Supplementary Table 1**). (**c**) Confocal microscopy of the location of *Igh* alleles relative to pericentromeric clusters representative of pre–B cells from wild-type, MiEκ-knockout and 3′Eκ-knockout mice. *Igh* probes: CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) in combination with a γ-satellite probe. Individual alleles from the same cell are shown in different sections. Original magnification, ×100. Data are representative of three experiments.

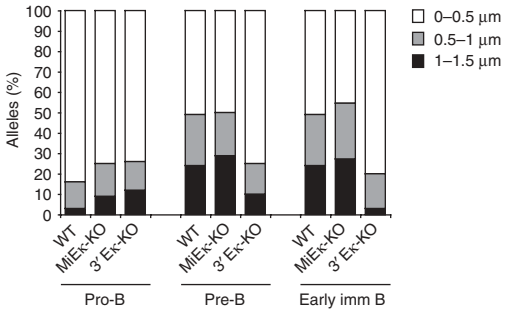

Figure 6 The association of *Igh* and *Igk* mediates decontraction of the *Igh* locus. Two-color three-dimensional DNA FISH analysis of the distances separating the V_HJ558 and C_H gene segments in cells sorted from wild-type, MiEκ-knockout and 3′Eκ-knockout bone marrow at various developmental stages. *Igh* probes: CT7-526A21 (V_HJ558) and CT7-34H6 (C_H). Data are representative of three experiments.

both alleles located in this repressive compartment (**Fig. 5a**). As in MiEκ-knockout pre–B cells, we noted no defect in *Igk* locus contraction in 3′Eκ-knockout pre–B cells (**Supplementary Table 5**). Because the absence of either enhancer had a similar effect on *Igk* locus localization, we expected to find that deletion of 3′Eκ would result in a greater frequency of association between *Igh* and *Igk* loci and more pericentromeric recruitment of the *Igh* locus. However, we obtained the opposite result (**Fig. 5** and **Supplementary Tables 1,2**). The extent of association between the two loci did not increase beyond the pro–B cell stage, and the *Igh* locus remained positioned away from pericentromeric clusters at the pre–B cell stage and was not repositioned to pericentromeric heterochromatin until the early immature B cell stage. Furthermore, pericentromeric recruitment of *Igh* at the immature B cell stage was not linked to interchromosomal association, as the two loci were positioned at different pericentromeric clusters in immature B cells. We repeated these experiments with *Igh* and *Igk* C-region probes to confirm that deletion of distal V gene regions did not affect the frequency of association of the two loci (**Supplementary Figure 1**). These results indicate that the 3′Eκ is important for the association between the *Igh* and *Igk* loci and that in the absence of *Igh-Igk* association, recruitment of the *Igh* locus to heterochromatin is delayed. This suggested that the *Igh* locus in 3′Eκ-knockout mice might remain accessible to the V(D)J recombinase during the pre–B cell stage of development.

Igh decontraction is mediated by association with *Igk*

Locus contraction mediated by looping is required for the rearrangement of distal and middle V_H gene segments to DJ_H segments, and subsequent decontraction at the pre–B cell stage of development contributes to allelic exclusion by preventing ongoing rearrangement of these V_H gene families[4,13]. Decontraction and pericentromeric recruitment occur at the pre–B cell stage of development, and, so far, observations suggest that these two processes occur simultaneously and may be linked[4]. Because pericentromeric recruitment of *Igh* is delayed and occurs in early immature B cells of 3′Eκ-knockout mice, independently of *Igk* association, we next sought to determine whether *Igh* locus decontraction was also affected by the absence of 3′Eκ. To address this, we measured the separation of the two ends of the *Igh* locus in sorted developing B cells from wild-type, MiEκ-knockout and 3′Eκ-knockout mice. We did two-color three-dimensional DNA FISH with the *Igh* probes CT7-526A21 and CT7-34H6, which map to the 5′ end of the *Igh* locus and the 3′ *Igh* C region, respectively (**Fig. 6** and **Supplementary Table 7** online). We grouped separation of the two ends of the *Igh* locus into the following three categories: 0–0.5 μm, 0.5–1 μm and 1–1.5 μm. Decontraction occurred in developing B cells from MiEκ-knockout mice, in which we documented *Igh-Igk* association and pericentric repositioning of *Igh*. In contrast, locus decontraction was impaired in 3′Eκ-knockout cells, in which *Igh-Igk* association was impaired and there was no substantial pericentric recruitment of *Igh* at the pre–B cell stage of development. Furthermore, *Igh* locus decontraction did not occur when *Igh* was repositioned to pericentromeric regions independently of the association of *Igk* in 3′Eκ-knockout immature B cells. These results suggest that *Igh* locus decontraction depends on both repositioning to pericentromeric regions and association with *Igk*.

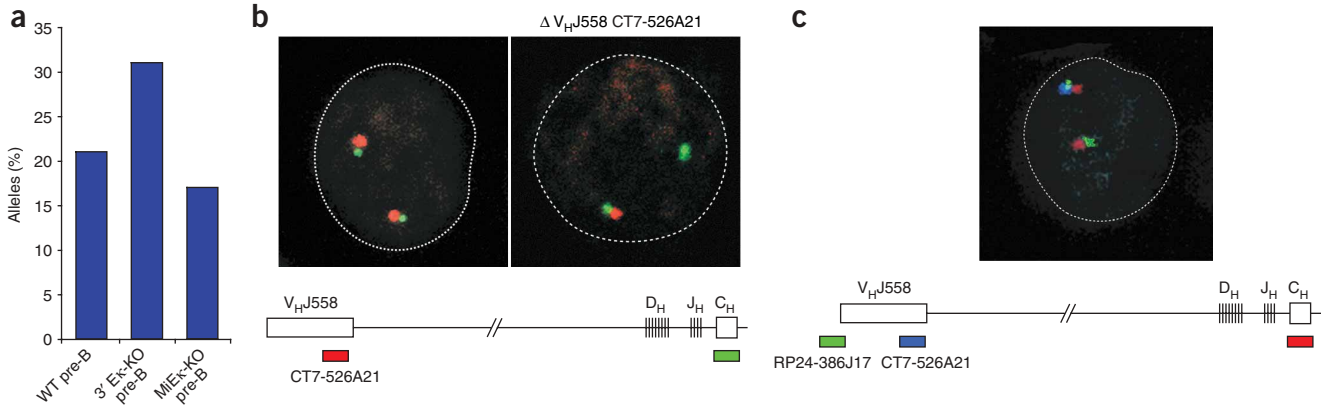

Figure 7 Deletion of the V_HJ558 CT7-526A1 probe occurs more frequently in 3′Eκ-knockout pre–B cells than in wild-type or MiEκ-knockout pre–B cells. Three-dimensional DNA FISH analysis with the *Igh* probes CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) in combination with the probe RP24-386J17, located outside the 5′ region of the *Igh* locus. (**a**) Frequency of deletion of the V_HJ558 CT7-526A1 signal in wild-type, 3′Eκ-knockout and MiEκ-knockout pre–B cells (**Table 1**, frequency of deletion of the V_HJ558 CT7-526A21 and RP24-386J17 signal). (**b**) Confocal microscopy (top row) showing the presence of both the CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) signals on both *Igh* alleles (left) and, in a different nucleus, the absence Δ of the CT7-526A21 (V_HJ558) signal on one allele (right). Below, positions and colors of probes. (**c**) Confocal microscopy (top row) showing the presence of CT7-526A21 (V_HJ558), CT7-34H6 (C_H) and RP24-386J17 signals on one *Igh* allele and the absence of the CT7-526A21 (V_HJ558) signal on the other allele. Below, position and colors of probes. Original magnification, ×100. Data are representative of three experiments.

Table 1 Signal deletion after rearrangement of V_H genes

Cell type	*Igh* Δ VHJ558 (CT7-526A21)	Δ 5′ probe (RP24-386J17)	Sample size
WT pro–B cell	9%	0%	86
3′Eκ-KO pro–B cell	9%	0%	152
MiEκ-KO pro–B cell	11%	ND	127
WT pre–B cell	21%	0%	146
3′Eκ-KO pre–B cell	31%	0%	253
MiEκ-KO pre–B cell	17%	ND	95

Frequency of deletion (Δ) of V_HJ558 CT7-526A21 and RP24-386J17 signals in wild-type, 3′Eκ-knockout and MiEκ-knockout pro–B cells and pre–B cells. ND, not done. Statistical analysis, **Supplementary Table 8** online.

Enhanced distal V_H-segment use in 3′Eκ-knockout cells

In our FISH analyses of developing B cells from wild-type, 3′Eκ-knockout and MiEκ-knockout mice, we used the probe CT7-526A21, which hybridizes to the 3′ end of V_HJ558 genes. Deletion of this bacterial artificial chromosome signal occurs after rearrangement of V_H genes located 5′ of this region. We noted such deletion in approximately 9% of wild-type and 3′Eκ-knockout pro–B cells and 11% of MiEκ-knockout pro–B cells. Deletion was higher in wild-type and MiEκ-knockout pre–B cells, at 21% and 17%, respectively, and it was further increased in 3′Eκ-knockout pre–B cells, at 31% (**Fig. 7a**,**b** and **Table 1**). As a control, in addition to the C-region probe CT7-34H6, we used a probe located outside of the 5′ end of the *Igh* locus, RP24-386J17. Signals from these two probes were always present, even in the absence of the CT7-526A21 signal (**Fig. 7c**). These data indicate that distal V_H gene rearrangement was greater in pre–B cells from 3′Eκ-knockout mice in which both *Igh* alleles remained in a contracted conformation and were positioned in euchromatic regions. This type of rearrangement would not be expected if the locus had undergone decontraction[4].

To verify that the differences in rearrangement at the pre–B cell stage are present in peripheral B cells, we analyzed the rearrangement status of the *Igh* locus in mature resting splenic B cells. We used PCR and Southern blot analysis to assess the relative amounts of proximal versus distal V_H-DJ_H rearrangements in wild-type, 3′Eκ-knockout and MiEκ-knockout mice. Distal *Igh* rearrangements were greater in 3′Eκ-knockout splenic B cells than in wild-type splenic B cells (**Fig. 8a**). Quantification of V_HJ558 rearrangements normalized to that of C_μ, which we used as a PCR control, showed that distal V_H rearrangement to the J_H3 gene segment was 50% higher in the absence of 3′Eκ (**Fig. 8b**). Rearrangement of the proximal V_H7183 family was slightly lower in 3′Eκ-knockout peripheral B cells, in particular those involving the J_H3 gene segment (**Fig. 8c**). Distal V_HJ558 rearrangements involving the J_H3 gene segment were 40% lower in MiEκ-knockout splenic B cells, whereas proximal V_H7183 rearrangements were mostly unchanged. Rearrangements to the middle V_H gene family V_HGam3.8 showed twofold more rearrangement of the furthest J_H3 gene segment in B cells lacking 3′Eκ than in wild-type B cells (**Fig. 8d**). These data indicate that in the absence of 3′Eκ, ongoing rearrangement at the pre–B cell stage of development changes the distribution of V_H rearrangement in mature B cells so that distal V_H gene rearrangement is greater.

DISCUSSION

Although it is known that regulatory elements in *Igh* and *Igk* are key to the promotion of accessibility to recombinase, little is known about how the accessibility of partially assembled DJ-rearranged *Igh* alleles is diminished and how they become refractory to the recombinase during the period of *Igk* rearrangement in pre–B cells. The interchromosomal associations we have noted here between *Igh* and *Igk* alleles suggest a mechanism for coordinating rearrangement and allelic exclusion at a stage in development when targeting of the recombinase is directed away from one locus and toward the other. Our study has demonstrated a previously unappreciated aspect of immunoglobulin gene regulation and indicates that association of two loci can induce conformational changes that could subsequently alter the function of these loci.

Published studies have shown that dynamic movement of immunoglobulin loci exerts control of recombination at many levels.

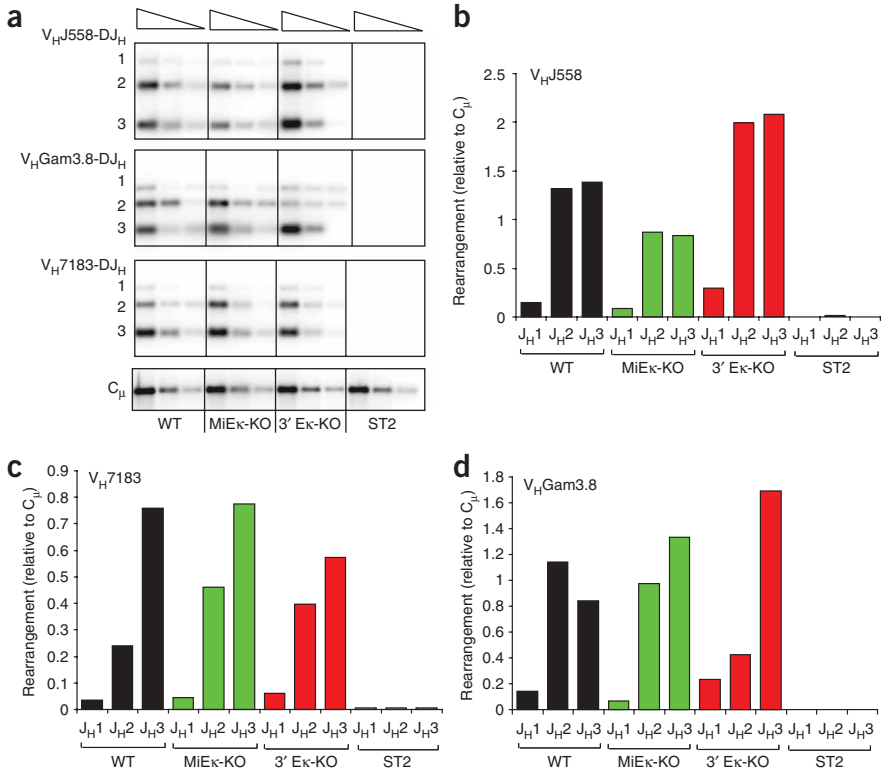

Figure 8 Altered spectrum of proximal versus distal V_H gene segment rearrangements in 3′Eκ-knockout splenic B cells. (**a**) PCR and Southern blot analysis of *Igh* rearrangements in $CD19^+$ splenic B cells (genotypes, below lanes; wedges above indicate serial threefold dilution of DNA samples). PCR primers detect V_HJ558 (top), V_HGam3.8 (middle) and V_H7183 (bottom) rearrangements to DJ_H1, DJ_H2 and DJ_H3 segments. Input DNA is normalized to amplification of a PCR fragment from the *Igh* Cμ region; ST2, DNA of stromal ST2 cells (negative control). Left margin, rearrangements involving J_H1, J_H2 and J_H3 gene segments. (**b**–**d**) Quantification of the rearrangements in **a**. The recombination signal is shown as a proportion of the C_μ amounts for each DNA dilution and as an average across the two highest dilutions of DNA. Data are representative of two experiments.

Enrichment of such loci at the nuclear periphery is important for restricting recombination to the B cell lineage[3]. Relocation to the center of the nucleus occurs at the pro–B cell stage of development and correlates with greater accessibility of the two loci[3,13,14].

Conformational changes involving contraction of the two loci occur after relocation. At the *Igh* locus, contraction mediated by looping correlates with recombination of distal and middle V_H gene rearrangement and may function to facilitate long-range recombination signal sequence interactions[4]. It is now known that all antigen receptor loci undergo similar conformational changes during recombination[15]. However, the mechanisms underlying locus contraction are not understood, although the transcription factor Pax5 and another unknown factor are required at the *Igh* locus[13]. It may be that the underlying mechanism regulating contraction of the *Igk* locus is similar to that of the *Igh* locus and that Pax5 could have a common function in this context. Our data obtained with the two *Igk* enhancer–knockout mice have indicated that individually, MiEκ and 3′Eκ are not required for locus contraction, as in the absence of either enhancer element, *Igk* loci adopted conformations similar to those seen in wild-type developing B cells. However, as the two enhancer elements have overlapping functions, we cannot rule out the possibility that the absence of both enhancer elements could affect this process.

At the pre–B cell stage of development, pericentromeric recruitment of one allele of *Igh* and *Igk* has been associated with the establishment of allelic exclusion[16]. The data we have reported here indicate that recruitment of the *Igk* locus is the initiating event and that it influences subsequent recruitment of the *Igh* locus. The *Igk* locus exerts its effect on the *Igh* locus through transient association at pericentromeric regions. Close association of *Igh* and *Igk* adds a further dimension to the control exerted by nuclear organization in regulating the process of recombination at these two loci. Our data indicate that *Igh* locus decontraction is dependent on association with the *Igk* locus at pericentromeric regions and that pericentromeric repositioning in the absence of *Igk* association does not induce decontraction. That conclusion is supported by our finding that locus decontraction did not occur in 3′Eκ-knockout immature B cells, in which delayed pericentromeric repositioning of *Igh* occurred independently of association with *Igk*. Thus, the association we noted in our FISH analysis may indeed be a physical interaction. It could be that repressive factors bound to the pericentromeric *Igk* allele are shared with the associated *Igh* allele and that this induces changes in chromatin that disrupt contraction. Such possibilities add a further layer of complexity to immunoglobulin gene regulation and indicate that association of the two loci brings about an epigenetic change to alter locus conformation.

Our microscopy-based investigations provided information about events in individual cells from sorted developmental populations. Because we used fixed cells, our analysis can provide only an incomplete picture of the dynamic movements of these loci. However, it is apparent from our studies here that immunoglobulin loci are not static. After they have repositioned to pericentromeric regions at the pre–B cell stage of development, they do not remain in the same nuclear location as the associated alleles do but move apart to different pericentromeric clusters at the immature B cell stage. The 'snapshot in time' that we analyzed is therefore probably a low estimate of the frequency with which close association of *Igh* and *Igk* occurs. This could also explain why only 60–70% of pre-B and immature cells had one *Igh* and one *Igk* allele repositioned to pericentromeric regions. In the remaining cells from those populations, where we found no pericentromeric recruitment, we noted that one allele was always located at the periphery (J.S., unpublished observations). It may be that alleles shuttle between the periphery and pericentromeric heterochromatin, two environments thought to be repressive.

Our analysis of cells lacking 3′Eκ or MiEκ identified a shared function for these regulatory elements in diminishing pericentromeric repositioning of the *Igk* locus, in line with their overlapping functions in activating transcription and rearrangement[7,8]. It is likely that binding of transcription factors to the two enhancers at the pre–B cell stage of development prevents association with centromeres and that a limited supply of activating factors in the cell could result in relocalization of one *Igk* allele to heterochromatic regions. Analysis of developing B cells from 3′Eκ-knockout mice identified a function for this regulatory element in mediating an interchromosomal *Igh-Igk* association that altered the conformation of the *Igh* locus at the pre–B cell stage of development. The absence of activating factors or the presence of repressive factors at the 3′Eκ region on the pericentromeric *Igk* allele may facilitate the binding of repressive factors at the *Igh* locus. Sharing of common repressive factors could mediate association of pericentromeric *Igk* with the nonproductive or DJ-rearranged *Igh* allele, thereby decreasing accessibility and altering the conformation of the latter.

If that model is correct, then locus decontraction should only take place on the pericentromeric *Igh* allele, mediated by interaction with 3′Eκ. This is difficult to conclude from analysis of contracted and decontracted alleles in developing B cells, as pericentromeric recruitment of immunoglobulin loci is a transient process, the percentage of alleles found at these regions is lower at later stages of development, and no pericentromeric recruitment of immunoglobulin loci is found in mature resting cells[11]. As a result, although contracted loci are identified mainly in euchromatic regions, decontracted alleles are also present in these locations.

Because we noted pericentromeric repositioning of the *Igh* locus at the immature B cell stage in 3′Eκ-knockout mice, it is likely that at later stages of development, additional mechanisms enforce silencing of the *Igh* locus. An alternative pathway probably involves a decrease in interleukin 7 and subsequent deacetylation, a modification known to decrease accessibility at the *Igh* locus[17]. Early repression of *Igh* by *Igk* at the pre–B cell stage of development is important for inducing locus decontraction. Interleukin 7 receptor signaling may act as an important 'backup' mechanism for decreasing accessibility of the *Igh* locus, which prevents ongoing rearrangement of distal V_H segments on the second DJ_H-rearranged *Igh* allele during *Igk* rearrangement.

These enhancers have overlapping functions and individually have been shown to be quantitatively important but not essential for *Igk* rearrangement. Before our study, to our knowledge the only functional difference identified for the two enhancers was involvement of MiEκ in regulating monoallelic demethylation of *Igk*[8]. Our studies have indicated that absence of the two enhancers has a very different effect on the *Igh* locus.

Our data showing greater deletion of the V_HJ558-associated signal in 3′Eκ-knockout cells but not in MiEκ-knockout cells suggest that the *Igh* locus can continue to undergo distal V_H gene rearrangement only when the *Igh* locus remains in a contracted conformation in euchromatic regions of the nucleus. Despite the alterations in *Igh* locus conformation noted in the 3′Eiκ-knockout pre–B cells, flow cytometry of allotypically 'marked' *Igh* alleles has failed to detect evidence of allelic inclusion (data not shown). Thus, additional mechanisms must exist to enforce *Igh* allelic exclusion.

Several studies have shown that transcription of genes located on one chromosome can be controlled by regulatory elements located on a different chromosome. These include an association between the promoter of the gene encoding the T helper type 1 cytokine

interferon-γ and the locus-control region of genes encoding T helper type 2 cytokines[18]. In addition, a nonallelic association has been reported between the 'imprinting' control region of the locus encoding the growth factor IGF-2 and fetal liver mRNA H19 and the locus encoding the ubiquitin ligase complex subunit WSB1 and the tumor suppressor neurofibromin, which is mediated by CTCF, a factor involved in DNA methylation[19]. Interchromosomal pairing has also been demonstrated between the two X chromosomes, and this association has been linked to silencing of the genes on one X chromosome during X-chromosome inactivation[20,21]. This contrasts with the interchromosomal associations involved in olfactory receptor 'choice', which may be important for promoting gene expression[22]. All those interchromosomal associations, including the one we have identified here, involve the coordination of 'decisions' involving changes in gene accessibility and/or expression. It could be that physical association of loci or alleles in the nucleus is essential for ensuring that the timing of coordinated changes in gene expression is correctly regulated. Published analyses of interchromosomal associations have not examined association relative to pericentromeric heterochromatin. Our analysis has indicated that association between *Igh* and *Igk* is a transient event that is important for early silencing of one *Igh* allele in pre–B cells and for changing the conformation of this allele. Thus, we have demonstrated here an interchromosomal association occurring at pericentromeric heterochromatin. The challenge now is to define the precise regions of *Igh* that mediate its association with *Igk* and to identify the proteins that mediate this association.

METHODS

Flow cytometry sorting and analysis. Antibodies to the following, conjugated to fluorescein isothiocyanate, phycoerythrin, allophycocyanin or phycoerythrin-indotricarbocyanine (all from BD Biosciences) or phycoerythrin–Alexa Fluor 610 (Invitrogen), were used for flow cytometry: CD19 (1D3), CD25 (IL-2Rα; PC61), CD117 (c-Kit; 2B8), IgM (II/411), IgD (11-26c.2a), CD4 (RM4-5), CD8α (53-6.7) and Thy-1.2 (53-2.1). Isolated bone marrow cells were stained with the appropriate antibody combination, and pro–B cells were isolated as $CD19^+$c-Kit^+IgM^- cells, pre–B cells were isolated as $CD19^+CD25^+IgM^-$ cells, early immature B cells were isolated as $CD19^+IgM^{lo}IgD^-$ cells, and late immature B cells were isolated as $CD19^+IgM^{hi}IgD^-$ cells, on a FACSAria (Becton Dickinson) or a MoFlo (Dako). The purity of the sorted cells was verified by reanalysis by flow cytometry.

Three-dimensional DNA FISH and confocal microscopy. Cells sorted by flow cytometry were washed three times in PBS and then were fixed on poly-L-lysine-coated slides for two-color and three-color three-dimensional DNA-FISH analysis as described[13]. Probes were directly labeled by nick translation with ChromaTide Alexa Fluor 488-5-dUTP, ChromaTide Alexa Fluor 594-5-dUTP (Molecular Probes) or dUTP-indodicarbocyanine (GE Healthcare). The γ-satellite probe was prepared from a plasmid containing eight copies of the γ-satellite repeat sequence[11] and was directly labeled with dUTP–fluorescein isothiocyanate (Roche; Enzo Biochem) or dUTP-indodicarbocyanine. Cells were analyzed by confocal microscopy on a Leica SP2 and Leica SP5 AOBS system (Acousto-Optical Beam Splitter). Optical sections separated by 0.3 μm were collected, and only cells with signals from both alleles (typically 90%) were analyzed.

For statistical analysis of the separation of locus probe signals or subnuclear location of probe signals, the χ^2 test was applied to observed and expected frequencies. The original data of cell numbers were used rather than percentages. Expected frequencies were calculated according to standard methods[23] and χ^2 probabilities were calculated in Microsoft Excel.

Activation of splenic B cells. Splenic B cell samples were purified by depletion of $CD43^+$ cells and cells were activated in culture with antibody to CD40 (10 μg/ml) as described[11].

V(D)J recombination analysis. Splenic B cells were isolated by $CD19^+$ magnetic-activated cell sorting and were digested with proteinase K. DNA was isolated by phenol extraction and ethanol precipitation. PCR analysis of immunoglobulin genes used published primers[13,24]. PCR cycle numbers were adjusted to be in the linear range, on the basis of analysis of serially diluted DNA. PCR products were separated by agarose gel electrophoresis, were transferred to Hybond-XL membranes and were analyzed by Southern blot with a published upstream J_H3 oligonucleotide probe or purified C_μ PCR product[13,24]. A phosphorimager and Quantity One software (Bio-Rad) were used for quantification of hybridization products. DNA amounts were normalized to those of C_μ. The average of the two highest dilutions of DNA is presented.

Mice. 3′Eκ-knockout mice were provided by F. Alt (Harvard University)[7]. MiEκ-knockout mice have been described[9]. All animal experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee of the New York University School of Medicine.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.L.H., bone marrow preparation, FISH experiments and V(D)J analysis; D.F., FISH experiments; K.M., bone marrow preparation and flow cytometry staining; E.C. and M.S.S., V(D)J analysis and breeding of 3′Eκ-knockout mice and MiEκ-knockout mice; H.-E.L., V(D)J analysis; Y.X., analysis of MiEκ-knockout mice; and J.A.S., FISH experiments and confocal analysis.

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