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Locus 'decontraction' and centromeric recruitment contribute to allelic exclusion of the immunoglobulin heavy-chain gene

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Abstract

Allelic exclusion of immunoglobulin genes ensures the expression of a single antibody molecule in B cells through mostly unknown mechanisms. Large-scale contraction of the immunoglobulin heavy-chain (*Igh*) locus facilitates rearrangements between *Igh* variable (V_H) and diversity gene segments in pro–B cells. Here we show that these long-range interactions are mediated by 'looping' of individual *Igh* subdomains. The *Igk* locus also underwent contraction by looping in small pre–B and immature B cells, demonstrating that immunoglobulin loci are in a contracted state in rearranging cells. Successful *Igh* recombination induced the rapid reversal of locus contraction in response to pre–B cell receptor signaling, which physically separated the distal V_H genes from the proximal *Igh* domain, thus preventing further rearrangements. In the absence of locus contraction, only the four most proximal V_H genes escaped allelic exclusion in immature μ-transgenic B lymphocytes. Pre–B cell receptor signaling also led to rapid repositioning of one *Igh* allele to repressive centromeric domains in response to downregulation of interleukin 7 signaling. These data link both locus 'decontraction' and centromeric recruitment to the establishment of allelic exclusion at the *Igh* locus.

The diverse antigen receptor repertoire of lymphocytes is generated by V(D)J recombination, which assembles the variable regions of immunoglobulin and T cell receptor genes from discontinuous variable (V), diversity (D) and joining (J) gene segments during B and T cell development[1,2]. These gene segments are flanked by recombination signal sequences that function as recognition sites for the V(D)J recombinase consisting of recombination activating gene 1 (RAG1) and RAG2 proteins. After pairing of two compatible recombination signal sequences, the RAG1-RAG2 complex introduces double-strand DNA breaks between the recombination signal sequences and flanking gene segments, followed by processing and religation of the DNA ends by repair factors of the nonhomologous end-joining machinery[1,2].

V(D)J recombination is tightly controlled in a lineage- and stage-specific way. Immunoglobulin and T cell receptor genes are rearranged only in B and T lymphocytes, respectively[1,2]. In the B lymphoid lineage, the immunoglobulin heavy-chain (*Igh*) locus undergoes rearrangements in pro–B cells before recombination of the genes encoding immunoglobulin light chains (IgL) in small pre–B and early immature B cells[1,2]. Moreover,

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D_H-J_H rearrangements precede V_H-DJ_H recombination in the *Igh* locus, whereas, among the two IgL genes, the *Igk* locus rearranges before the *Igl* locus[3]. The observed temporal order of V(D)J recombination is determined mainly by the accessibility of the different gene loci and segments to the V(D)J recombinase[4,5], which is controlled at multiple levels, including sub-nuclear relocation[6], DNA demethylation[7], chromatin remodeling[8], histone acetylation[9,10] and germline transcription[4] of the different immunoglobulin loci.

The approximately 200 V_H genes of the *Igh* locus are spread over a 2.4-megabase region and can be divided into 15 distal, central or proximal V_H gene families according to their sequence similarity and position relative to the proximal D_H segments[11]. In non–B lymphoid cells and lymphoid progenitors, the two *Igh* alleles are present in an extended conformation at the potentially repressive periphery of the nucleus[6], where they are anchored via the distal V_HJ558 gene region with the proximal *Igh* domain facing toward the center of the nucleus[12]. This orientation of the *Igh* locus is likely to facilitate activation of the proximal domain in lymphoid progenitors, thus resulting in D_H-J_H rearrangements[10,13]. Early pro–B cell development is characterized by relocation of the *Igh* alleles to central nuclear positions[6], histone acetylation of the distal V_HJ558 genes in response to interleukin 7 (IL-7) signaling[10], antisense transcription along the entire V_H gene cluster[14] and long-range contraction of the *Igh* locus[6,12], which ultimately results in V_H-DJ_H recombination. The transcription factor Pax5 has an essential function in regulating contraction of the *Igh* locus[12.] The central and distal V_H genes are fully accessible in active chromatin and yet fail to rearrange in *Pax5*$^{-/-}$ pro–B cells[15] because of the physical separation of these genes from the proximal DJ_H-rearranged domain in the absence of locus contraction[12].

Successful rearrangement of the *Igh* locus leads to cell surface expression of the μ protein as part of the pre–B cell receptor (pre-BCR), which functions as an important checkpoint to signal proliferative expansion of populations of large pre–B cells, to induce subsequent differentiation to small pre–B cells and to establish allelic exclusion at the second, DJ_H-rearranged *Igh* allele[3,16,17]. Feedback inhibition of *Igh* recombination by the membrane-bound μ protein (referred to as allelic exclusion) was initially noted in mice expressing a μ transgene, which efficiently prevents V_H-DJ_H rearrangements at both endogenous *Igh* alleles during B cell development[18–21]. RAG protein expression is rapidly lost after pre-BCR signaling, which halts all further V(D)J recombination and ‘prepares the ground’ for the establishment of allelic exclusion in large pre–B cells[22]. Pre-BCR signaling also leads to histone deacetylation and thus reduced accessibility of the V_H genes in small pre–B cells, which has been considered as a possible feedback mechanism underlying allelic exclusion[23]. These chromatin alterations, however, could be an indirect consequence of pre-BCR signaling, as they depend on the pre-BCR-induced down-regulation of IL-7 signaling[23]. It is therefore still unknown what changes occur on the DJ_H-rearranged *Igh* locus during the short recombinase-free ‘window’ in large pre–B cells so that this allele is unable to further rearrange after subsequent reexpression of the RAG proteins in small pre–B cells.

Here we have identified, using fluorescence *in situ* hybridization (FISH), two previously unknown mechanisms that are likely to establish allelic exclusion during the recombinase-free transition phase in large pre–B cells. ‘Decontraction’ of both *Igh* loci was initiated in large pre–B cells and was maintained at all subsequent developmental stages. The reversal of *Igh* locus contraction is likely to prevent V_H-DJ_H rearrangements in small pre–B cells, in analogy to the extended *Igh* conformation in *Pax5*$^{-/-}$ pro–B cells[12]. Pre-BCR signaling simultaneously induced rapid repositioning of one *Igh* allele to repressive centromeric domains. This monoallelic centromeric recruitment was transiently maintained in small pre–B and early immature B cells, where it kept the nonfunctional *Igh* allele in an inactive state during IgL gene rearrangements. In contrast to the *Igh* alleles, the *Igk* gene underwent locus contraction specifically in small pre–B and immature B cells, thus demonstrating the general principle that

immunoglobulin loci are in a contracted state only in rearranging B lymphocytes. Furthermore, 'looping' of individual subdomains was responsible for the contraction of the *Igh* and *Igk* loci. Finally, both endogenous *Igh* alleles were in an extended state without having undergone centromeric recruitment in pre–B and immature B cells of μ-transgenic mice. In the absence of locus contraction, only the four most proximal V_H genes escaped allelic exclusion in μ-transgenic B lymphocytes. These data strongly suggest that both locus decontraction and centromeric recruitment establish allelic exclusion at the *Igh* locus in response to pre-BCR signaling.

RESULTS

Decontraction of the *Igh* locus in early pre–B cells

To study the contraction state of the *Igh* locus throughout B cell development, we used two-color three-dimensional DNA FISH analysis to localize the distal V_HJ558 and proximal $C_{\gamma1}$ gene segments in three-dimensionally preserved nuclei using confocal laser-scanning microscopy[12,24]. We isolated B lymphocytes of the following developmental stages by cell sorting: c-Kit$^+$CD19$^+$ bone marrow pro–B cells[25], which we cultured on ST2 cells in the presence of IL-7; B220$^+$CD25$^+$ bone marrow pre–B cells[25], which we visually identified as large cycling or small resting pre–B cells after FISH analysis; and splenic IgM$^+$ B cells, which we cultured in activating conditions[24]. We analyzed the sorted B lymphocyte populations using three-dimensional FISH with a labeled $C_{\gamma1}$ probe in combination with a V_HJ558 probe (Fig. 1a) that does not cross-hybridize with members of other V_H gene families[12]. The results of these three-dimensional FISH experiments are presented as confocal images (Fig. 1b) and as a statistical evaluation of the distances between the proximal $C_{\gamma1}$ and distal V_HJ558 gene segments of the *Igh* alleles (Fig. 1c). Consistent with previous results[6,12], most of the pro–B cells undergoing *Igh* recombination had their *Igh* loci in a contracted conformation (Fig. 1b), as the proximal and distal domains were separated in only 10% and 1% of the analyzed *Igh* alleles by a distance of 0.3–1 μm or 1–1.5 μm, respectively (Fig. 1c and **Supplementary Table 1** online). In contrast, the large pre–B cells mainly contained the *Igh* locus in an extended state (Fig. 1b), as there was a distance of 0.3–1 μm or 1–1.5 μm separating the V_HJ558 and $C_{\gamma1}$ genes in 33% and 30% of all *Igh* alleles, respectively (Fig. 1b,c). All subsequent developmental stages contained the *Igh* locus in a similarly extended state (Fig. 1b,c). Therefore, *Igh* alleles undergo decontraction after the production of a functional rearrangement at the onset of pre–B cell development.

The V_HJ558 gene family at the distal end of the *Igh* locus comprises about 44% of all *Igh* V_H genes11 and should give rise to the least extension after decontraction in response to productive V_H-DJ_H recombination, which juxtaposes the V_HJ558 gene family next to the $C_{\gamma1}$ region within a short distance of 100 kilobases (ref. 11) that cannot be resolved by three-dimensional DNA FISH analysis[12]. The signals of the V_HJ558 and $C_{\gamma1}$ genes were colocalized in 37–45% of the *Igh* alleles analyzed in pre–B cells. Large pre–B cells ('preB-II' cells)[26] and small pre–B cells (fraction D)[27] carry V_HJ558-DJ_H rearrangements on 46% and 41% of their *Igh* loci, respectively. The close correlation between the frequencies of colocalized signals and $V_HJ558DJ_H$-rearranged *Igh* loci strongly suggests that the distance separating the V_HJ558 and $C_{\gamma1}$ genes reflects the recombination status of the decontracted *Igh* alleles in pre–B cells. Accordingly, rearrangements involving genes from the middle of the V_H gene cluster are likely to fall within the category of 0.3- to 1.0-μm gene separation. Moreover, *Igh* alleles with proximal V_HQ52, V_H7183 and DJ_H rearrangements should give rise to the full extent of separation (1–1.5 μm), as noted before for the *Igh* alleles in *Rag2*$^{-/-}$*Pax5*$^{-/-}$ pro–B cells, which are unable to undergo *Igh* locus contraction and V(D)J recombination[12].

Thus, our data demonstrate that the contraction of the *Igh* locus is reversible and occurs only during V(D)J recombination in pro–B cells. Decontraction in response to pre-BCR signaling

is therefore likely to prevent further distal and central V_H gene recombination in pre–B cells, whereas it may still be compatible with proximal $V_H$gene rearrangements on the extended *Igh* locus, analogous to the contraction-deficient *Pax5*$^{-/-}$ pro–B cells[12].

Igk locus contraction coincides with recombination

The *Igk* locus contains approximately 140 V_κ genes spread over a 3-megabase region[28,29] and is thus as large as the *Igh* locus. The question therefore arises as to whether transient contraction of the *Igk* locus also facilitates V_κ-J_κ recombination by promoting the interaction between distal V_κ and proximal J_κ gene segments. Although a few V_κ-J_κ rearrangements take place early in pro–B cells[30], efficient recombination occurs in small resting pre–B cells and continues in early immature B cells[31] undergoing receptor editing[32]. We therefore divided the immature B cell population of the bone marrow by flow cytometry into early ($B220^+IgM^{lo}$) and late ($B220^+IgM^{hi}$) immature B cells (**Supplementary Fig. 1** online) and used these cells together with bone marrow pro–B and pre–B cells and activated splenic B cells to determine the contraction state of the *Igk* locus at various developmental stages. For this three-dimensional DNA FISH analysis, we used probes detecting the distal V_κ24 gene family and proximal C_κ gene segment at either end of the *Igk* locus (Fig. 2a). The results of these FISH experiments indicated that the V_κ24 and C_κ genes were separated by a large distance of 1–1.5 μm in 85% of the *Igk* alleles of pro–B and large cycling pre–B cells (Fig. 2b,c and **Supplementary Table 1** online). In contrast, the V_κ24 and C_κ signals were colocalized in most small pre–B cells (90%) and early immature B cells (78%). Late immature B cells were characterized by a notable increase in partially separated (0.3–1.0 μm) or widely separated (1–1.5 μm) *Igk* alleles (24% and 32%, respectively). In splenic B cells, the V_κ24 and C_κ signals were once again separated by a large distance (1–1.5 μm) in most *Igk* alleles (72%). The higher proportion of fully extended *Igk* (72%) versus *Igh* (31%) alleles noted in splenic B cells (Figs. 1c and 2c) is explained by the different outcome of V(D)J recombination at the two immunoglobulin loci. As all V_H genes are oriented in the same transcriptional direction as the D_H and J_H segments[11], recombination invariably results in deletion of the intervening sequences at the *Igh* locus. In contrast, about two thirds of all V_κ genes are present in antisense orientation relative to the J_κ segments[29], which leads to inversion and thus retention of the intervening sequence after *Igk* recombination. Thus, our data unequivocally demonstrate that *Igk* alleles are present in a contracted state only when they undergo V_κ-J_κ recombination in small pre–B and immature B cells. Hence, a general principle emerges demonstrating that the contraction of *Igh* and *Igk* loci is reversible and occurs only in cells actively undergoing V(D)J recombination.

Locus contraction by looping

We next took advantage of three-color three-dimensional DNA FISH to further analyze the conformation of *Igh* and *Igk* loci in cells in which locus contraction occurs (Fig. 3). For these experiments, we used a third probe detecting the V_H11 or V_κ21 gene, which are located between the probes used in previous FISH experiments (Fig. 3a,c). DNA FISH analysis of *Rag2*$^{-/-}$ pro–B cells with V_HJ558, V_H11 and $C_{\gamma1}$ probes identified several *Igh* alleles (n = 24) for which the signals of the three probes contacted each other in an order that did not reflect their linear arrangement on the *Igh* locus (Fig. 3b and **Supplementary Fig. 2** online). For example, the V_HJ558 signal was located in the middle between the V_H15 and $C_{\gamma1}$ signals in the *Igh* allele in Figure 3b, demonstrating that the V_HJ558 gene region looped back onto the proximal *Igh* domain. Likewise, DNA FISH analysis of early immature B cells identified *Igk* alleles (n = 20) for which the relative arrangement of the V_κ24, V_κ21 and C_κ signals was incompatible with their gene order in the *Igk* locus (Fig. 3c,d and **Supplementary Fig. 2** online). The *Igk* allele in Figure 3d showed a looping configuration that brought the proximal C_κ domain in contact with both the V_κ24 and V_κ21 gene families. These data indicate that looping mediates

long-range interactions between distinct domains of the *Igh* or *Igk* loci, thus resulting in locus contraction.

Centromeric location of one *Igh* allele in pre–B cells

The transcriptionally silent *Igh* allele is located at centromeric heterochromatin in activated splenic B cells, whereas the productively rearranged, expressed *Igh* allele is positioned away from centromeric clusters[24]. To determine whether centromeric recruitment of one *Igh* allele may contribute to allelic exclusion in pre–B cells, we examined the nuclear position of *Igh* loci relative to centromeres at various developmental stages using three-color three-dimensional DNA FISH (Fig. 4 and **Supplementary Table 2** online). We used a γ-satellite probe to visualize the centromeric foci, whereas V_HJ558 and $C_{\gamma1}$ probes localized the distal and proximal *Igh* domains within the nucleus. There was infrequent centromeric recruitment (16%) in pro–B cells, as reported before[24] (Fig. 4c). In contrast, we detected association of one *Igh* allele with γ-satellite clusters in 66–75% of large and small pre–B cells and in 65% of early immature B cells (Fig. 4a,c). During subsequent development, the frequency of centromeric recruitment was decreased to 34% in late immature B cells (Fig. 4c) and to 28% in resting splenic B cells[24] (Fig. 5). Hence, centromeric recruitment of one *Igh* allele is initiated together with allelic exclusion at the onset of pre–B cell development and is transiently maintained in B lymphocytes undergoing IgL gene rearrangements.

To gain insight into whether the DJ_H-rearranged or nonproductively V_HDJ_H-rearranged *Igh* allele is recruited to centromeres in pre–B cells, we assessed individual *Igh* alleles for both centromeric recruitment and locus decontraction. For this, we evaluated all cells that showed centromeric recruitment and additionally contained one *Igh* allele with widely separated (1–1.5 μm) V_HJ558 and $C_{\gamma1}$ genes. The widely separated *Igh* allele in this cell population was recruited to the centromere in 81–91% of small and large pre–B cells and in 88% of activated splenic B cells (Fig. 4d). The class of widely separated *Igh* loci is likely to consist of all *Igh* alleles with proximal V_H7183-DJ_H, V_HQ52-DJ_H and D_H-J_H rearrangements, whereas the recombination of V_H genes located in the central and distal *Igh* domains should give rise to only partial gene separation (0–1 μm) after decontraction in pre–B cells. The rearrangement status of *Igh* alleles was analyzed before in large pre–B (preB-II) cells by single-cell PCR assay[26]. Reevaluation of those data demonstrated that D_H-J_H and nonfunctional V_H-DJ_H rearrangements accounted for 81% of all rearrangements within the proximal *Igh* domain compared with 26% of nonfunctional V_H gene rearrangements in the central and distal *Igh* domains (**Supplementary Table 3** online). The close correlation between centromeric recruitment (81–91%) and nonproductive recombination (81%) in the proximal *Igh* domains strongly suggests that the DJ_H-rearranged or nonfunctionally V_HDJ_H-rearranged *Igh* allele is recruited to the centromere at the onset of pre–B cell development.

IL-7 signaling prevents centromeric *Igh* recruitment

As shown by three-dimensional FISH analysis, the recruited *Igh* locus was oriented at the centromere in pre–B and activated B cells in such a way that the distal V_HJ558 gene family was positioned closer to the γ-satellite cluster than the proximal V_H7183 and $C_{\gamma1}$ genes in 96% (50 of 52) and 90% (102 of 113) of recruited *Igh* alleles, respectively (Fig. 4a,b and data not shown). Centromeric recruitment of the V_HJ558 gene family thus coincides with histone deacetylation of the distal *Igh* domain in pre–B cells[23], suggesting a link between these two processes. Deacetylation of the distal V_H genes has been associated with downregulation of IL-7 receptor signaling at the pro–B cell–to–pre–B cell transition[23]. Moreover, the distal V_HJ558 genes are hypoacetylated in resting splenic B cells, but can be acetylated during B cell activation in response to IL-7 signaling[23], which mimics opening of the distal *Igh* domain in early pro–B cell development[10]. To investigate whether IL-7 signaling prevents centromeric recruitment of the *Igh* locus, we activated splenic B cells for 4 d with an antibody to CD40

(anti-CD40) in the presence or absence of IL-7, as described[23]. DNA FISH analysis with $C_{\gamma 1}$ and γ-satellite probes showed that B cell activation in the presence of IL-7 resulted in substantially reduced centromeric recruitment of the *Igh* locus (38%), which approximated the basal recruitment (28%) found in resting B cells at day 0 (Fig. 5a,b and **Supplementary Table 4** online). The observed inverse correlation between IL-7 signaling and centromeric *Igh* recruitment in splenic B cells thus suggests that the downregulation of IL-7 receptor signaling in large pre–B cells[23,33] may be required for the initiation of centromeric recruitment of the *Igh* locus at the onset of pre–B cell development. Treatment of activated B cells with IL-7, however, had no effect on repositioning of the *Igk* allele[24] to centromeric clusters (Fig. 5a,b), indicating that different factors regulate centromeric recruitment at the *Igh* and *Igk* loci. In activated splenic B cells, the *Igk* locus was recruited to the centromeres also via the distal $V_{\kappa}24$ region (**Supplementary Fig. 3** online), demonstrating that centromeric repositioning of immunoglobulin loci via their distal domain is a more general phenomenon.

Extended *Igh* alleles in μ-transgenic B lymphocytes

Cell surface expression of a functional μ protein is essential for initiating of feedback inhibition of V_H-DJ_H recombination and *Igh* allelic exclusion in pre–B cells[3,16]. Allelic exclusion of the *Igh* locus has been studied extensively in μ-transgenic mice, in which the endogenous *Igh* alleles rarely undergo V_H-DJ_H rearrangements, in contrast to efficient D_H-J_H recombination[18–21]. To further investigate the relationship between centromeric recruitment, locus decontraction and V_H-DJ_H recombination, we next analyzed the pre–B and B cells of two μ-transgenic strains of mice, M54 (ref. 34) and MD4 (ref. 35). Flow cytometry analysis confirmed that the splenic B cells of M54 and MD4 mice expressed transgenic IgM^a and no endogenous IgM^b (C57BL/6), as published[36,37] (Fig. 6a and data not shown). Before three-dimensional FISH analysis, we tested the V_HJ558 and $C_{\gamma 1}$ probes on metaphase spreads of activated splenic MD4 B cells to ensure that they detected only the endogenous *Igh* loci at the telomeres of chromosome 12 instead of the μ transgene (Fig. 6b and data not shown). Contrary to expectations, the three-dimensional DNA FISH experiments failed to show any substantial association of the endogenous *Igh* loci with γ-satellite clusters in bone marrow pre–B and immature B cells and activated splenic B cells of either M54 or MD4 mice (Fig. 6c–e and **Supplementary Table 2** online). In addition, three-color FISH analyses of pre–B and immature B cells showed that the $C_{\gamma 1}$ signals were separated from the V_HJ558 signals by a distance of 1–1.5 μm in more than 84% of all endogenous *Igh* alleles (Fig. 6d,f and **Supplementary Table 4** online). Hence, this situation resembles the noncontracted state of the germline *Igh* alleles in *Rag2*$^{-/-}$*Pax5*$^{-/-}$ pro–B cells[12]. We conclude therefore that the endogenous *Igh* alleles of μ-transgenic B lymphocytes are present in an extended conformation and undergo allelic exclusion in the absence of centromeric recruitment.

Allelic inclusion of the most proximal V_H genes

The data reported above suggest that decontraction rather than centromeric recruitment is responsible for allelic exclusion of endogenous *Igh* alleles in μ-transgenic pre–B cells. The absence of locus contraction furthermore suggests that proximal V_H genes should be able to rearrange in μ-transgenic pre–B cells in analogy to *Pax5*$^{-/-}$ pro–B cells[12]. Indeed, mouse B cells expressing a human μ transgene have been shown to carry proximal V_HQ52 gene rearrangements at a relatively high frequency[20], although the results obtained with the mouse M54 transgene are more ambiguous[37]. We therefore reinvestigated the rearrangement status of endogenous *Igh* alleles in splenic M54 B cells, which we sorted for expression of the μ^a transgene (**Supplementary Fig. 4** online). Rearrangements of the V_HQ52 and V_H7183 genes were readily identified in M54 B cells, albeit at a lower frequency than in wild-type B cells and *Pax5*$^{-/-}$ pro–B cells (Fig. 7). However, we were unable to detect V_HGam3.8 and V_HJ558 gene rearrangements in M54 B cells (Fig. 7), consistent with the fact that these genes depend on *Igh* locus contraction for efficient recombination[12]. Hence, our results, together with earlier

work[20], unequivocally demonstrate that proximal V_H genes fail to be allelically excluded in B lymphocytes expressing either a mouse or a human μ transgene.

As the two overlapping V_HQ52 and V_H7183 gene families constitute the proximal 320-kilobase region of the V_H gene cluster[11] (Fig. 8a), the question arises as to whether all of these genes or only a subset of them are able to undergo V_H-DJ_H recombination in the absence of *Igh* locus contraction. To investigate this, we compared the spectrum of V_HQ52-DJ_H and V_H7183-DJ_H rearrangements in the presence of *Igh* locus contraction in wild-type pro–B cells[12] and in the absence of *Igh* locus contraction in *Pax5*$^{-/-}$ pro–B cells[12], M54 transgenic IgM^a B cells and '*Ik*$^{Pax5/+}$' pro–T cells[12], which ectopically express a *Pax5* 'mini-gene' under the control of the Ikaros (*Zfpn1a1*) locus[38]. We amplified V_H-DJ_H4 rearrangements of pro–B and pro–T cells and V_H-DJ_H3 rearrangements of M54 (V_HDJ_H4)[34] transgenic B cells by PCR, then cloned and sequenced them and assigned the sequences to the various members of the V_HQ52 and V_H7183 gene families (Fig. 8b,c). In the presence of locus contraction, V_H genes across the entire 320-kilobase region underwent V_H-DJ_H recombination in wild-type pro–B cells, although with different efficiencies, as exemplified by the published high rearrangement frequency of the $V_H7183.b2$ (V_H81X) gene[39,40]. In contrast, only the two most proximal, functional V_H genes of the V_HQ52 (b1 and b2) and V_H7183 (b2 and b4) gene families were able to rearrange at a high frequency in M54 B lymphocytes and *Ik*$^{Pax5/+}$ pro–T cells, whereas *Pax5*$^{-/-}$ pro–B cells showed the same trend of preferentially rearranging the more proximally located V_H gene (Fig. 8b,c). These data indicate that only the four most proximal V_H genes of the *Igh* locus efficiently escape allelic exclusion in μ-transgenic B lymphocytes.

DISCUSSION

Allelic exclusion is responsible for monoallelic expression of immunoglobulin genes, which ensures the single-receptor specificity of B cells, and yet the molecular mechanisms underlying this important phenomenon are still mostly 'enigmatic'[3]. Here we have described centromeric recruitment and locus decontraction as two previously unknown mechanisms that contribute to allelic exclusion of immunoglobulin genes. Locus decontraction seems to be particularly important, as all V_H genes, with the notable exception of the four most proximal V_H genes, were allelically excluded in the absence of *Igh* locus contraction in μ-transgenic B lymphocytes.

Centromeric recruitment has been associated with transcriptional silencing of the nonfunctionally rearranged *Igh* allele in activated B cells[24]. Here we have shown that centromeric repositioning of one *Igh* allele was initiated by the onset of pre–B cell development and was transiently maintained in small pre–B and immature B cells undergoing IgL rearrangements. The close statistical correlation between fully extended *Igh* alleles at the centromere and alleles with D_H-J_H and nonfunctional V_H-DJ_H rearrangements in the proximal *Igh* domain strongly indicated that centromeric recruitment contributes to the inactivation of the nonproductively rearranged *Igh* allele in pre–B and immature B cells. As the mechanism controlling the monoallelic repositioning to centromeres discriminates between the two *Igh* alleles, the question arises as to which distinguishing feature of these alleles determines the choice of the nonproductively rearranged *Igh* locus for centromeric recruitment. For example, 40% of all pre–B cells contain one productive and one nonproductive V_H-DJ_H rearrangement[3]. These two alleles functionally differ from each other only by a single frameshift mutation resulting in transcripts with a premature termination codon, which could be discriminated in the nucleus by the 'nonsense-mediated' mRNA decay pathway[41]. As transcription, mRNA processing and monitoring of the nonsense codon take place in the proximal *Igh* region, it is conceivable that this information is already transmitted in the contracted state to the distal V_HJ558 gene region, which subsequently is recruited to centromeric clusters in pre–B cells. The *Igk* locus is also associated with centromeres via the distal $V_κ24$ region in activated splenic B cells, indicating that the centromeric recruitment of

immunoglobulin loci via their distal domain is a more general phenomenon. During the pro–B cell–to–pre–B cell transition, the distal V_HJ558 genes are not only recruited to centromeres but also undergo histone deacetylation, leading to reduced chromatin accessibility[23] as a consequence of the downregulation of IL-7 receptor signaling in pre–B cells[23,33]. The idea of a causal link between histone deacetylation and centromeric recruitment of the *Igh* locus is supported by the fact that treatment of activated splenic B cells with IL-7 interferes with centromeric recruitment of the *Igh* allele (as reported here) while simultaneously inducing histone acetylation of the distal V_HJ558 genes[23]. These findings are in agreement with the temporal coincidence of histone deacetylation and centromeric recruitment during the establishment of heritable silencing at the terminal deoxynucleotidyltransferase (*Dntt*) locus[42]. It is unclear, however, whether histone deacetylation is the cause or consequence of subnuclear repositioning to centromeres, which constitute an environment with abundant histone deacetylase complexes[43]. Finally, centromeric recruitment seems to be incomplete in pre–B cells, as only 66–75% of all nonfunctional *Igh* alleles are positioned at centromeres. However, the *Igh* alleles in the remaining approximately 30% of cells were located at the nuclear periphery[12] (data not shown), which may function, in addition to the centromeres, as a repressive subnuclear compartment[44].

In contrast to centromeric recruitment, locus decontraction equally affects the two *Igh* alleles at the onset of pre–B cell development, as it disconnects the proximal region from the central-distal domains on both *Igh* loci. This physical separation, however, has a functional consequence only for the incompletely DJ_H-rearranged *Igh* allele, where rearrangements of central and distal V_H genes are probably prevented, analogous to the situation described for *Pax5*$^{-/-}$ pro–B cells[12,15]. Whereas the *Igh* locus is in a decontracted state in pre–B cells and all subsequent developmental stages, the *Igk* locus specifically undergoes contraction during the phase of *Igk* rearrangements in small pre–B and immature B cells. Hence, distinct factors must be involved in controlling the contraction of *Igh* and *Igk* loci at different stages of B lymphopoiesis. Our FISH analyses demonstrated the new general principle that immunoglobulin loci actively undergo contraction only in rearranging cells, whereas they are in the extended default state at all other developmental stages. Our experiments furthermore showed that the looping of multiple subdomains is responsible for contraction of both the *Igh* and *Igk* loci in rearranging B lymphocytes. At the molecular level, Pax5 has been identified as a key regulator, which induces *Igh* locus contraction in pro–B cells in cooperation with an unknown factor 'X'[12]. The inactivation of Pax5, however, is unlikely to be the cause of locus decontraction in response to pre-BCR signaling, as the function of this transcription factor is required throughout B cell development[45]. Identification of factor 'X' will be needed to test whether pre-BCR signaling induces the loss of this regulator, thereby leading to decontraction of the *Igh* locus.

Immunoglobulin μ transgenes have been used successfully to study allelic exclusion of the endogenous *Igh* alleles during B cell development[18–21]. Our analysis of two different transgenes (M54 and MD4) has unequivocally demonstrated that the endogenous *Igh* alleles are present in an extended conformation but are not recruited to centromeres in transgenic pre–B and immature B cells. The absence of centromeric recruitment raises the question of how relevant the use of immunoglobulin μ transgenes is for investigating the phenomenon of allelic exclusion. Indeed, early expression of a functional μ transgene is known to considerably shorten or even bypass pro–B cell development[46], in which both *Igh* loci are normally made accessible at the chromatin level[4,5,10] and undergo locus contraction[6,12] before V_H-DJ_H recombination. As μ-transgenic B cell precursors have only very low expression of V_HJ558 germline transcripts (data not shown), the distal V_H gene region may never be reorganized into accessible acetylated chromatin, which, however, seems to be a prerequisite for subsequent centromeric recruitment of the *Igh* locus in pre–B cells. Our experiments using B cells from μ-transgenic mice thus suggest a function for centromeric recruitment in reducing the accessibility of

central-distal domains of *Igh* loci that have previously been opened up during proB cell development. In contrast, the proximal *Igh* domain is activated in both wild-type as well as μ-transgenic pre–B and immature B cells, as germline transcription of the proximal V_H7183 genes is as efficient in these cells as in pro–B cells (data not shown), and D_H-J_H recombination of endogenous *Igh* loci occurs at a high rate in transgenic pre–B cells[21]. Unexpectedly, however, only the four most proximal V_H genes were efficiently rearranged in transgenic B cells, indicating that the absence of locus contraction interferes with V_H-DJ_H recombination of all other V_H genes in *Igk*-rearranging pre–B and immature B cells. In wild-type mice, the rare allelically included B cells, which express two different IgM proteins on their cell surface, show a disproportionately high frequency of proximal V_HQ52-DJ_H and V_H7183-DJ_H rearrangements[47]. Given our results, it is conceivable that the most proximal V_H genes escaping allelic exclusion may give rise to some of these V_H-DJ_H rearrangements not only in pro–B cells but also in small pre–B and immature B cells.

In summary, our study has provided insight into how pre-BCR signaling controls allelic exclusion at the *Igh* locus. Pre-BCR signaling results in the rapid loss of RAG protein expression, thereby halting all further V(D)J recombination and ‘preparing the ground’ for the establishment of allelic exclusion[22]. However, it is not known which changes the *Igh* locus would undergo during the short recombinase-free ‘window’ so that it could no longer be rearranged after subsequent re-expression of RAG proteins. As shown here, pre-BCR signaling in large cycling pre–B cells additionally induces rapid decontraction and centromeric recruitment of the *Igh* locus, which are likely to prevent V_H-DJ_H rearrangement of the second *Igh* allele after re-expression of the V(D)J recombinase in small pre–B cells.

METHODS

Mice

Ik$^{Pax5/+}$, *Pax5*$^{+/-}$ and *Rag2*$^{-/-}$ mice[38,48,49] and μ-transgenic M54 and MD4 mice[34,35] were maintained on the C57BL/6 background and were genotyped as described[34,35,38,48,49].

Flow cytometry sorting and analysis

Antibodies to the following, coupled to fluorescein isothiocyanate (FITC), phycoerythrin or allophycocyanin, were used for flow cytometry: B220 (RA3-6B2), CD4 (L3T4), CD8a (53-6.7), CD19 (1D3), CD25/IL-2Rα (PC61), CD117/c-Kit (2B8), IgM^a (DS1) and IgM^b (AF6-78; PharMingen), and goat polyclonal anti-mouse IgM (Southern Biotech). Wild-type bone marrow was stained with the appropriate antibody combination, and pro–B cells were isolated on a MoFlo cell sorter (Dako-Cytomation) as c-Kit$^+$CD19$^+$ cells; pre–B cells, as B220$^+$CD25$^+$ cells; early immature B cells, as B220$^+$ IgM^{lo} B cells; and late immature B cells, as B220$^+$IgM^{hi} cells. The purity of the sorted cells was verified by flow cytometry reanalysis (**Supplementary Fig. 1** online).

Activation of splenic B cells

Splenic B cells of BALB/c mice were isolated by elimination of non–B cells by magnetic cell sorting with anti-CD43 MACS beads (Miltenyi Biotec) and then were activated with anti-CD40 (FGK-45) as described[24]. The activated B cells were grown with or without 1% conditioned supernatant of IL-7–producing J558L cells[48] and were collected after 4 d for three-dimensional DNA FISH analysis.

Probes for FISH

The locus-specific DNA probes were prepared from the published bacterial artificial chromosomes CT7-526A21 (V_HJ558)[6], RP24-282021 (V_H11), CT7-296M13 ($V_κ$24)[50] and

CT7-113G24 ($V_\kappa 21$)[51] and plasmids HE17 ($C_{\gamma 1}$) and IgkC (C_κ)[24] and were labeled by nick translation with dUTP-indodicarbocyanine, digoxygenin-dUTP or biotin-dUTP (Roche/Enzo Biochem). The γ-satellite probe was prepared from a plasmid containing eight copies of the γ-satellite repeat sequence[24] and was labeled directly with dUTP-rhodamine or dUTP-indodicarbocyanine (Amersham Pharmacia).

Three-dimensional DNA FISH and confocal analysis

Cells sorted by flow cytometry were washed three times in PBS and then were fixed onto poly-L-lysine-coated slides for two- and three-color three-dimensional DNA FISH analysis as described in detail[12,24,52]. Digoxygenin-labeled DNA probes were detected with sheep rhodamine-coupled anti-digoxygenin (Roche/Enzo Biochem), followed by further signal amplification with Texas red–coupled anti-sheep (Vector). Biotinylated DNA probes were detected with FITC-avidin followed by further signal amplification with biotinylated FITC-coupled anti-avidin and FITC-avidin (Vector). Cells were analyzed by confocal microscopy on a Leica SP2 AOBS (acoustica optical beam splitter) system. Optical sections separated by 0.3 μm were collected, and only cells with signals from both alleles (typically 90%) were analyzed.

V(D)J recombination assay

$B220^+$c-Kit^+ pro–B cells were sorted from the bone marrow of 2-week-old *Pax5*$^{-/-}$ mice; $B220^+$ splenocytes, from wild-type mice; and IgM^a $CD19^+$ splenocytes, from M54 transgenic mice at the age of 4.5 months (**Supplementary Fig. 4** online). DNA was isolated and analyzed for the presence of V_H-DJ_H rearrangements (Fig. 7) by PCR assay with published primers as described[12].

PCR cloning and sequencing of V_H-DJ_H rearrangements

Pax5$^{+/+}$ and *Pax5*$^{-/-}$ bone marrow pro–B cells as well as *Ik*$^{Pax5/+}$ $CD4^+CD8^+$ thymocytes were isolated by flow cytometry sorting as described[12], followed by DNA preparation and PCR amplification of V_HQ52-DJ_H4 and V_H7183-DJ_H4 rearrangements with the following primers[27]:5′-CTCACAGAGCCTGTCCATCAC-3′ (forward V_HQ52 V_HB), 5′-GTGGAGTCTGGGGGAGGCTTA-3′ (forward V_H7183 V_HE) and 5′-TCTCAGCCGGCTCCCTCAGGG-3′ (reverse J_H4A). IgMa $CD19^+$ splenocytes were isolated from 4.5-month-old M54 mice as shown in **Supplementary Fig. 4** online. As the μ transgene M54 carries a J_H4 rearrrangement[34], we amplified the rearrangements of endogenous *Igh* alleles from M54 transgenic B cells using the forward V_H7183 V_HE or V_HQ52 V_HB primer in combination with the J_H3 primer[12] 5′-GTCTAGATTCTCACAAGAGTCCGATA-GACCCTGG-3′. The V_H-DJ_H4 or V_H-DJ_H3 PCR fragment was gel-purified and was cloned into the pGEM-T easy vector (Promega), followed by DNA sequencing of individual clones. The 250– to 300–base pair 3′ sequence of each V_H gene was assigned by sequence comparison to its corresponding V_HQ52 and V_H7183 family member. For this purpose, we annotated the entire V_HQ52 and V_H7183 gene region of the mouse C57BL/6 strain (http://mendel.imp.univie.ac.at/SEQUENCES/VH/) based on the *Igh* sequences of the mouse genome database (www.ensembl.org; release NCBIm32). Only V_H sequences with distinct VDJ joints were used for statistical analysis, with identical sequences being counted only once.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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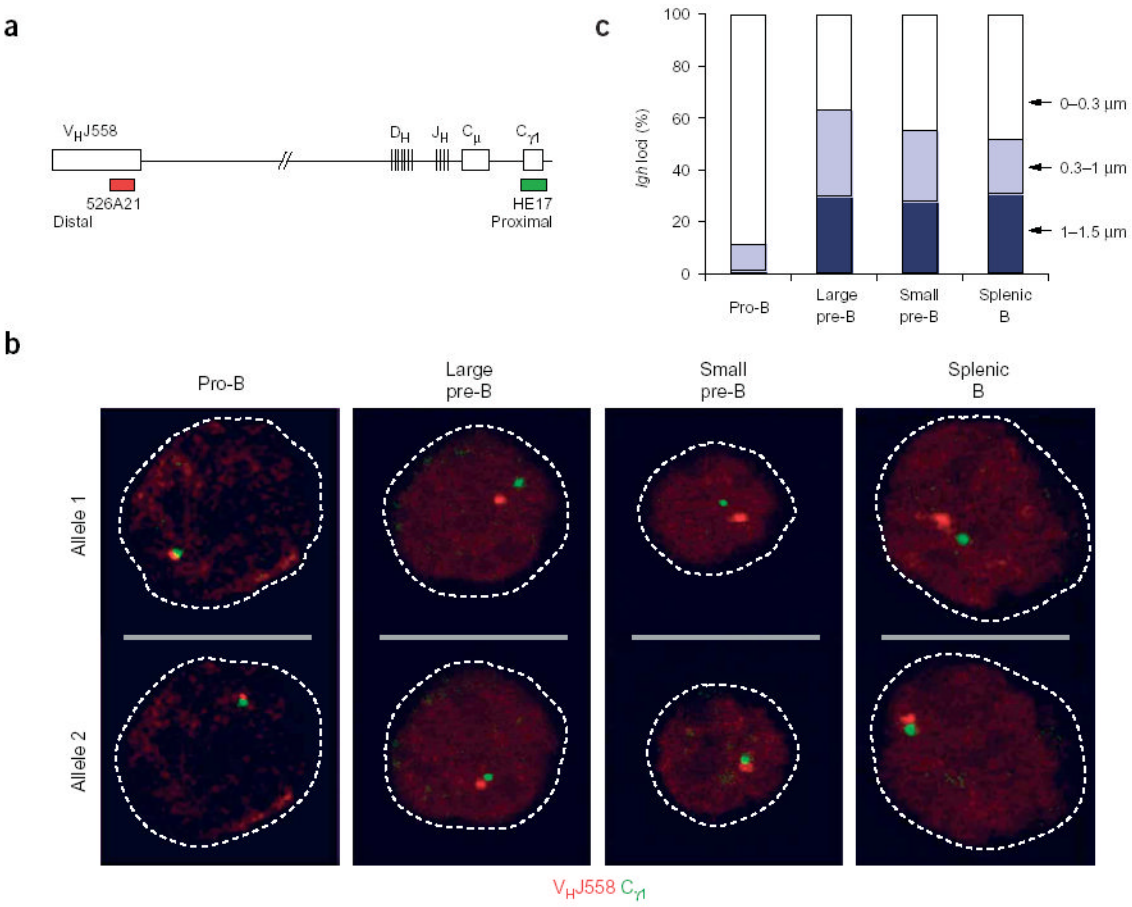

Figure 1.

Decontraction of the *Igh* locus in large pre–B cells. (**a**) *Igh* locus (not drawn to scale), indicating the positions of bacterial artificial chromosome (BAC) 526A21 (ref. 12) and plasmid HE17 (ref. 24), which were used to generate the V_HJ558 (red) and $C_{\gamma1}$ (green) probes, respectively. (**b**) Representative confocal sections through the nuclei of B lymphocytes at various developmental stages (above images) in which three-dimensional DNA FISH analysis of the *Igh* locus was done with V_HJ558 (red) and $C_{\gamma1}$ (green) probes. The two *Igh* alleles of each cell are presented on separate optical sections. Broken lines outline the contours of the nuclei. Pre–B cells were identified as large or small cells under the microscope. (**c**) Separation of V_HJ558 and $C_{\gamma1}$ gene segments. The distance (in μm) separating the V_HJ558 and $C_{\gamma1}$ segments was evaluated statistically for B lymphocytes of various developmental stages (horizontal axis). Actual numbers and sample sizes are in **Supplementary Table 1** online.

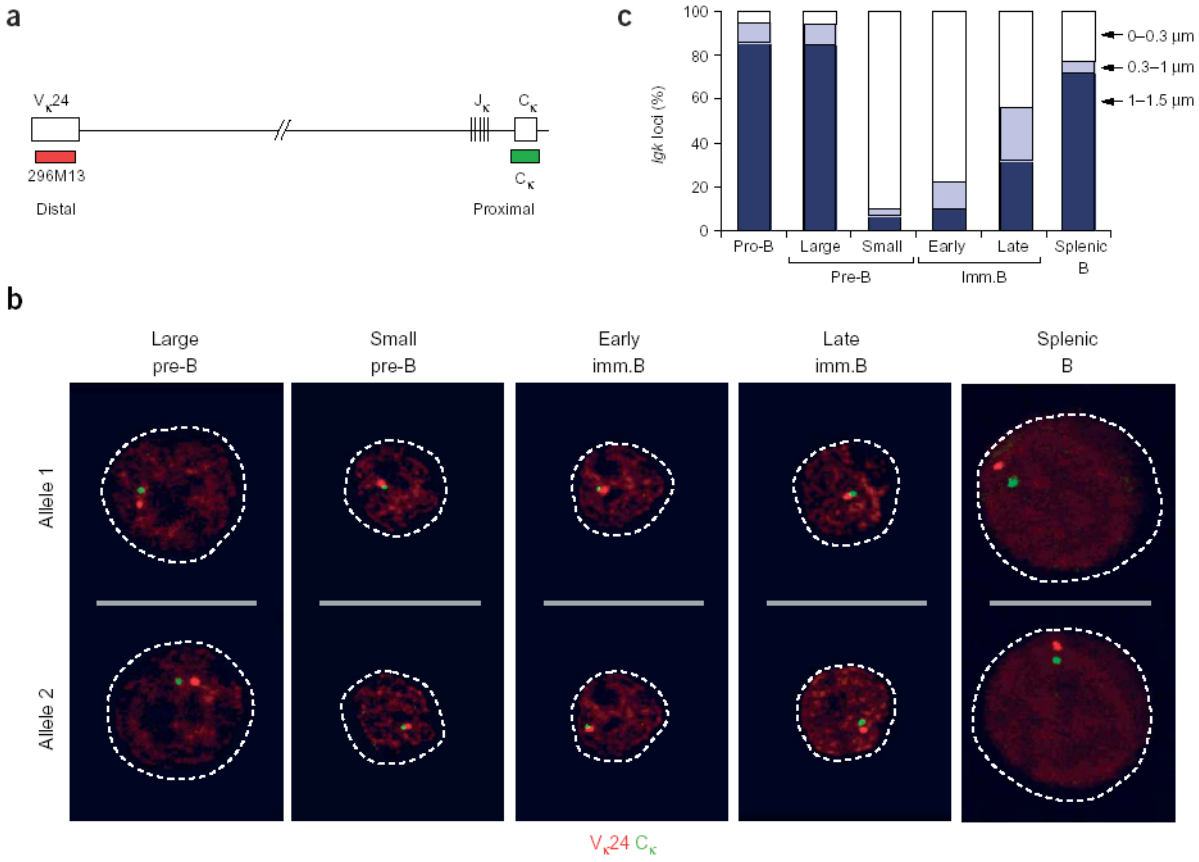

Figure 2.

Contraction and decontraction of the *Igk* locus. (**a**) *Igk* locus (not drawn to scale), indicating the positions of BAC 296M13 (ref. 50) and plasmid IgkC^{24}, which were used to generate the V_κ24 (red) and C_κ (green) probes, respectively. (**b**) Representative confocal sections through B cells at various stages of development (above images) in which three-dimensional DNA FISH analysis of the *Igk* locus was done with V_κ24 (red) and C_κ (green) probes. The two *Igk* alleles of each cell are presented in separate optical sections. Flow cytometry sorting of the developmental stages is in **Supplementary Fig. 1** online. (**c**) Separation of V_κ24 and C_κ gene segments. The distance (in μm) separating the V_κ24 and C_κ segments was evaluated statistically for cells of various developmental stages (horizontal axis). Actual numbers and sample sizes are in **Supplementary Table 1** online. Imm.B, immature B cell.

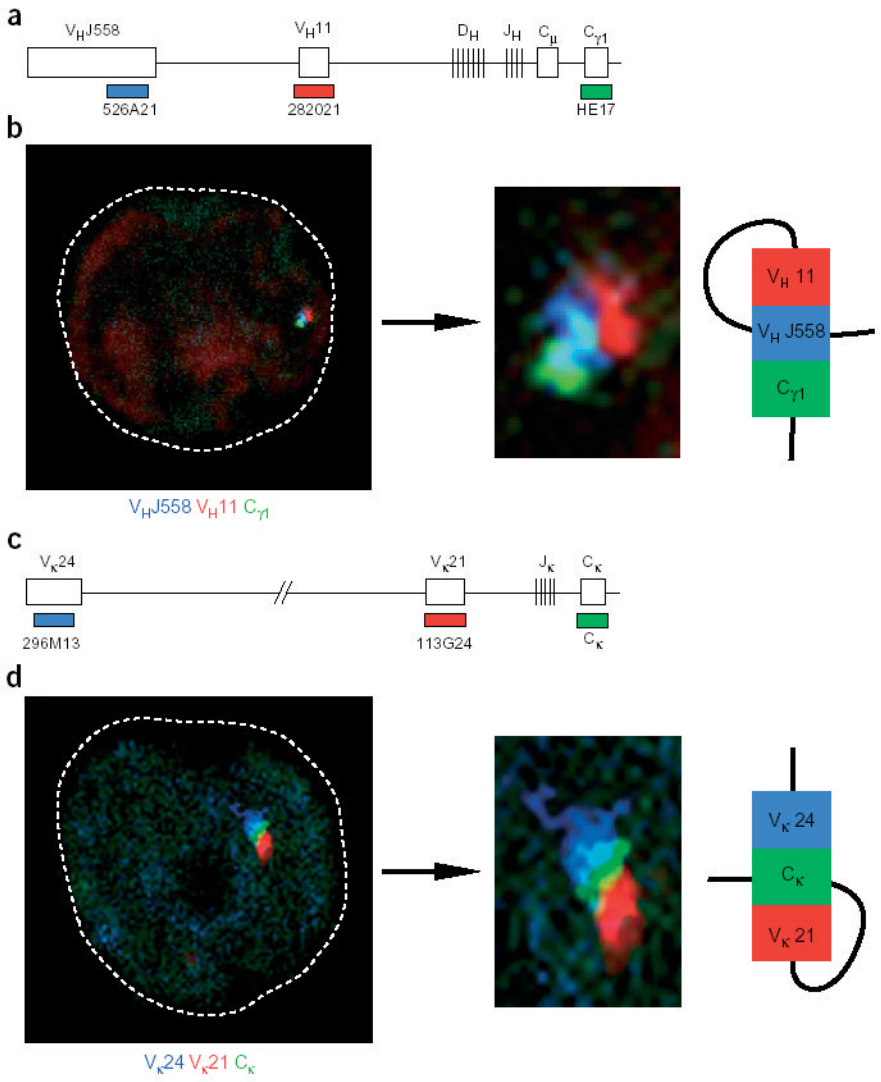

Figure 3.

Contraction by looping of the *Igh* and *Igk* loci. (**a**) *Igh* locus, indicating the position of the third V_H11 probe (BAC 282021). (**b**) Confocal section through the nucleus of a *Rag2*$^{-/-}$ pro–B cell, which was analyzed by three-dimensional DNA FISH with V_HJ558 (blue), V_H11 (red) and $C_{\gamma1}$ (green) probes. Middle, enlargement of the *Igh* allele shown at left; right, looping configuration. (**c**) *Igk* locus, indicating the position of the third $V_\kappa21$ probe (BAC 113G24; ref. [51]). (**d**) Confocal image of an early immature B cell showing the relative positions of the $V_\kappa24$ (blue), $V_\kappa21$ (red) and C_κ (green) probe signals. Middle, higher magnification of the *Igk* allele shown at left; right, looping configuration. There was similar looping of *Igk* and *Igh* loci in 20 and 24 cells, respectively. Additional alleles with *Igk* and *Igh* looping are in **Supplementary Fig. 2** online.

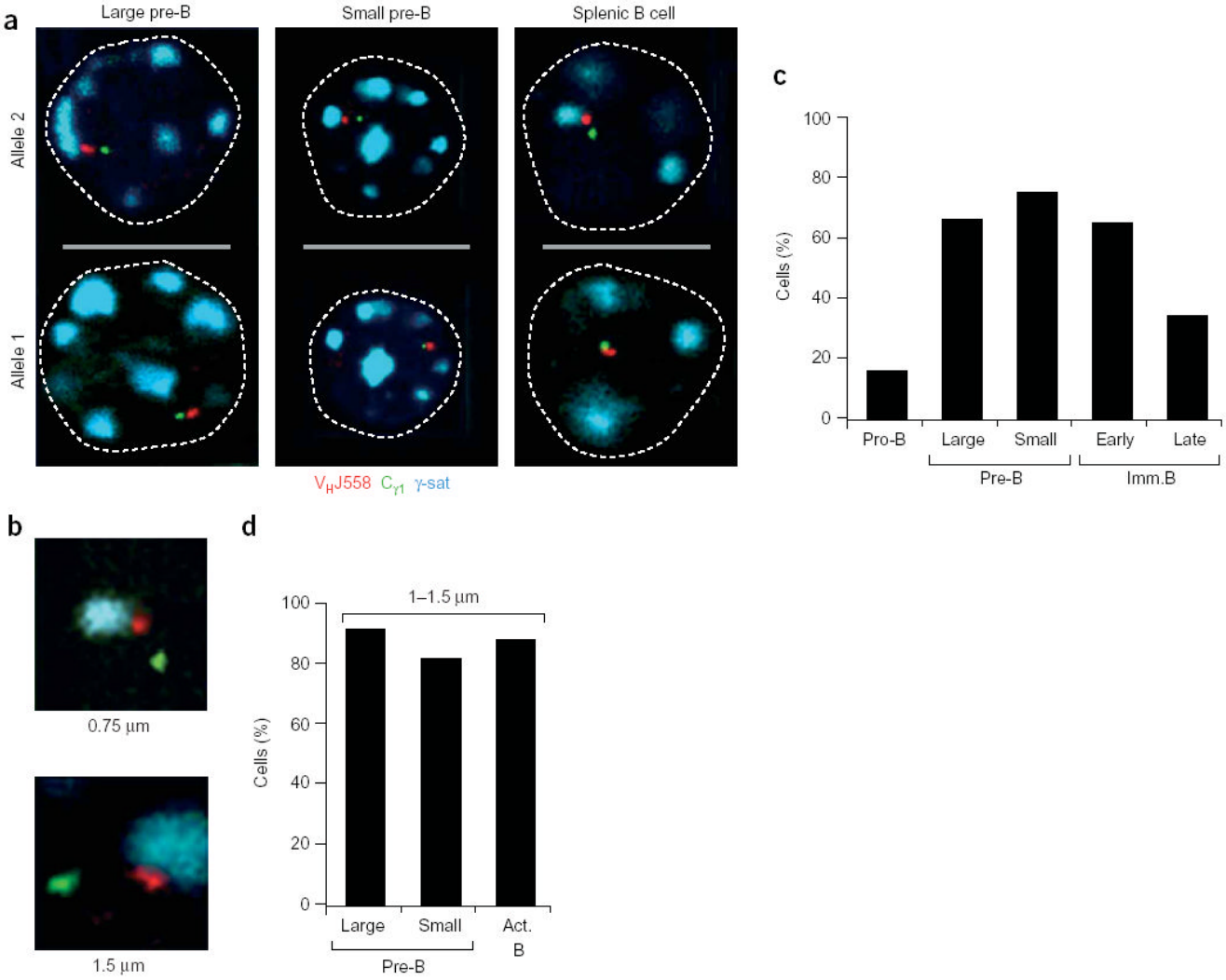

Figure 4.

Monoallelic centromeric recruitment of the *Igh* locus during B cell development. (**a**) Centromeric location of one *Igh* allele in bone marrow pre–B cells. Large and small pre–B cells and activated splenic B cells were analyzed by three-color three-dimensional DNA FISH with V_HJ558 (red), $C_{\gamma 1}$ (green) and γ-satellite (γ-sat; blue) probes. The relative positions of the three signals are shown in confocal sections through the nuclei of these cells. (**b**) Association of the distal V_HJ558 gene domain with centromeric clusters. Enlargements show the orientation and decontraction of the *Igh* locus at γ-satellite clusters. Below images, distance between the V_HJ558 and $C_{\gamma 1}$ probe signals. (**c**) Monoallelic recruitment of the *Igh* locus to centromeres. Data represent the percentage of cells showing association of one *Igh* allele with centromeric heterochromatin in various B cell developmental stages, sorted as indicated in **Supplementary Fig. 1** online. Actual numbers and sample sizes are in **Supplementary Table 2** online. (**d**) Preferential location of widely separated *Igh* alleles at the centromeres. The cells showing monoallelic centromeric recruitment were subdivided into a population of cells containing an *Igh* allele with wide separation (1–1.5 μm) of the V_HJ558 and $C_{\gamma 1}$ genes. Data represent the percentage of centromeric recruitment of the widely separated *Igh* allele in this cell population for large and small pre–B cells and activated (Act.) splenic B cells.

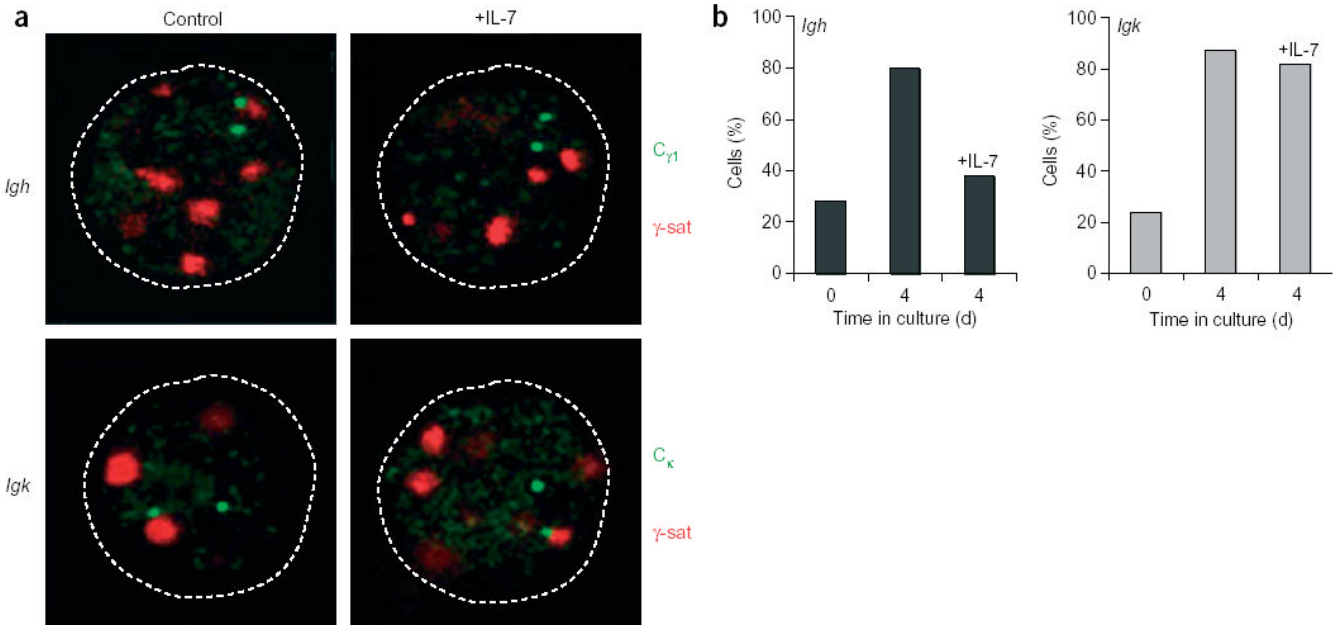

Figure 5.

IL-7 signaling prevents centromeric recruitment of the *Igh* locus in splenic B cells. (**a**) Two-color three-dimensional DNA FISH analysis of activated splenic B cells with (+IL-7) or without (Control) IL-7, showing the proximity of the $C_{\gamma 1}$ (*Igh*) and C_{κ} (*Igk*) signals (green) with γ-satellite clusters (red). Both *Igh* or *Igk* alleles are on the same optical section. (**b**) Statistical analysis of cells with centromeric association of one *Igh* or *Igk* allele in splenic B cells before and after *in vitro* activation with anti-CD40 in the presence (+IL-7) or absence of IL-7. Actual numbers and sample sizes are in **Supplementary Table 4** online.

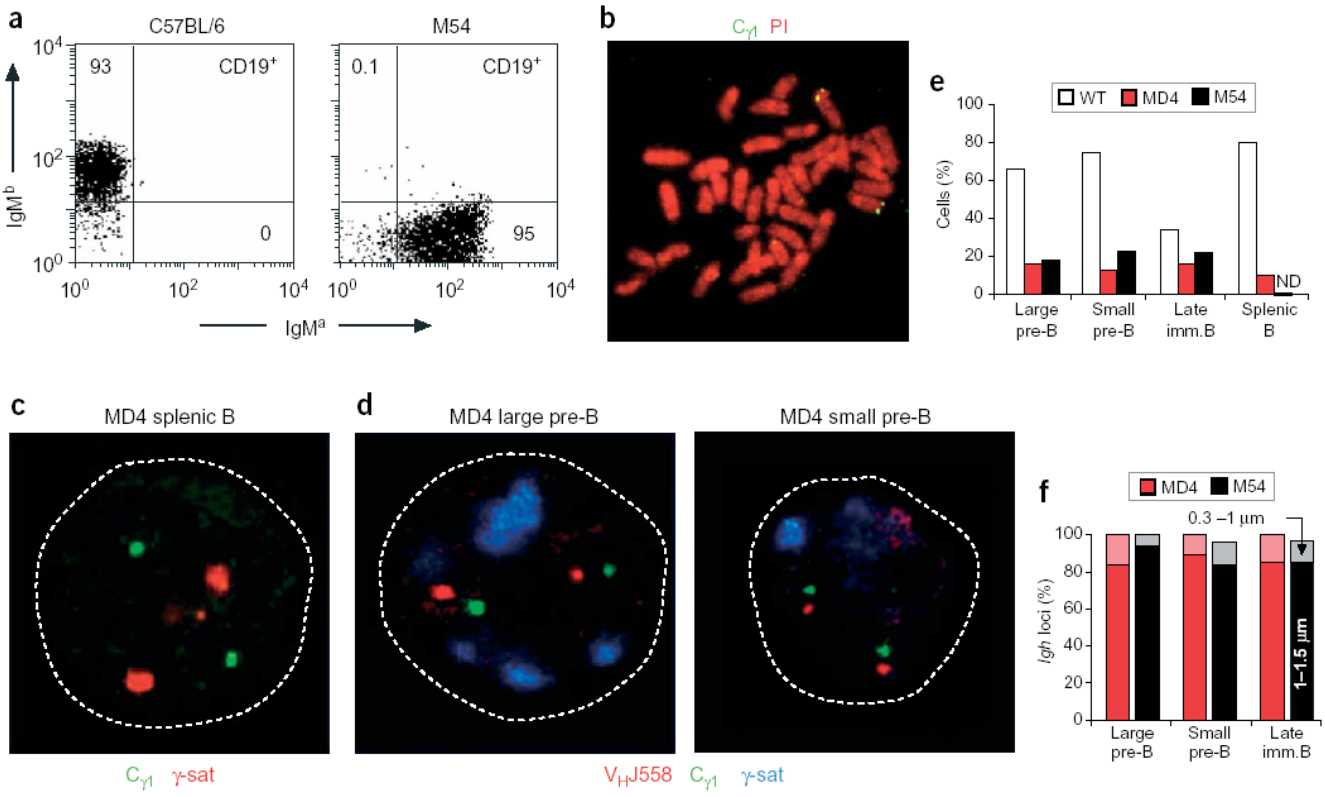

Figure 6.

Absence of centromeric recruitment and locus contraction of endogenous *Igh* alleles in μ-transgenic B cells. (**a**) Flow cytometry of IgM expression on $CD19^+$ B cells of C57BL/6 and M54 transgenic mice. The M54 transgene of the IgM^a haplotype was derived from the BALB/c hybridoma 17.2.25 (ref. 34) and was crossed into the C57BL/6 mouse strain of the IgM^b haplotype. Lymph node B cells from a 3-week-old M54 mouse express transgenic IgM^a but no endogenous IgM^b, as published[36,37]. Numbers in dot plots indicate the percentages of cells in quadrants. The same degree of allelic exclusion was noted for MD4 transgenic B cells[35] (data not shown). (**b**) Metaphase spread of MD4 transgenic B cells. Specific hybridization of the $C_{\gamma1}$ probe (green) is detected at the telomeres of chromosome 12. Chromosomes were counterstained with propidium iodide (PI; red). (**c**) Confocal section through the nucleus of an activated splenic MD4 B cell. The $C_{\gamma1}$ regions (green) of both endogenous *Igh* alleles are not associated with γ-satellite clusters (red), as visualized by three-dimensional DNA FISH. (**d**) Confocal sections through the nuclei of large and small pre–B cells of the MD4 transgenic mouse. The $C_{\gamma1}$ (green) and V_HJ558 (red) signals are widely separated and are not associated with γ-satellite clusters (blue). (**e**) Statistical analysis of the monoallelic centromeric association of endogenous *Igh* loci in B lymphocytes of wild-type (WT) and MD4 and M54 transgenic mice. ND, not determined. Actual numbers and sample sizes are in **Supplementary Table 2** online. (**f**) Statistical analysis of the distance separating the distal V_HJ558 and proximal $C_{\gamma1}$ regions of the endogenous *Igh* loci in developing MD4 (red) and M54 (black) transgenic B lymphocytes (**Supplementary Table 4** online). Light or dark shading indicates V_HJ558-$C_{\gamma1}$ gene separation of 0.3–1 μm or 1–1.5 μm, respectively.

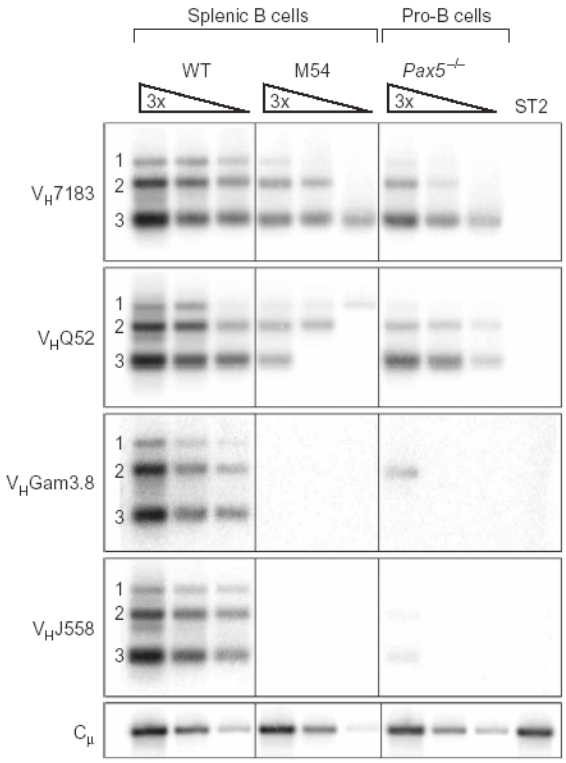

Figure 7.

Proximal V_H-DJ_H rearrangements in M54 transgenic B cells. IgMa $CD19^+$ B cells expressing the μa transgene were isolated by flow cytometry sorting from the spleens of M54 transgenic mice (**Supplementary Fig. 4** online). PCR was used to determine the frequency of the different V_H-DJ_H rearrangements in these transgenic B cells versus *Pax5*$^{-/-}$ bone marrow pro–B cells and wild-type $B220^+$ splenocytes. Threefold (3×) serial DNA dilutions were analyzed by PCR with V_H family–specific forward primers and a J_H3 reverse primer[12], which was unable to amplify the V_HDJ_H4-rearranged M54 transgene[34]. Bottom, input DNA was normalized by PCR amplification of an *Igh* C_μ fragment; far right lane, DNA of stromal ST2 cells (negative control). Numbers along the left margin indicate rearrangements to the J_H1, J_H2 and J_H3 segments. The same result was obtained in two independent experiments.

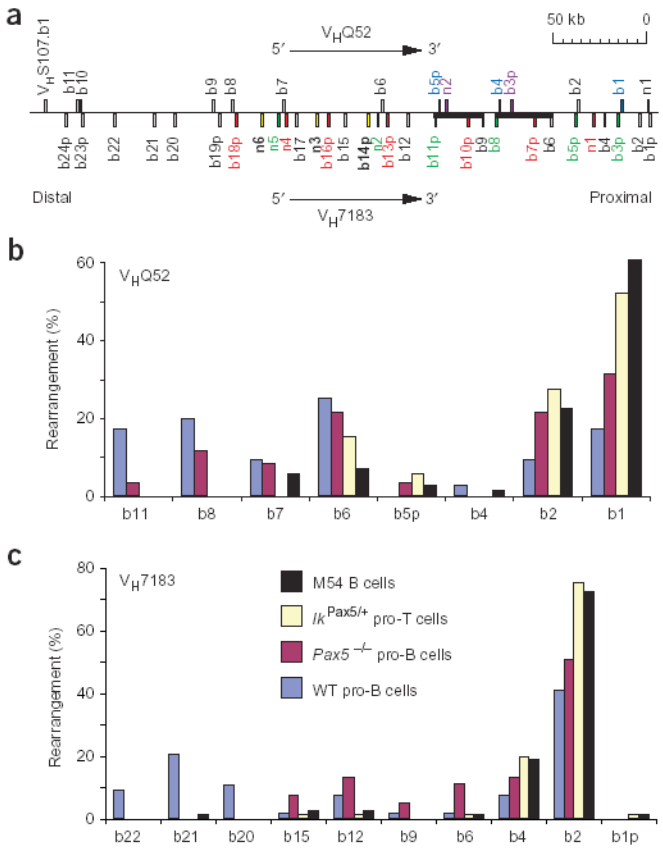

Figure 8.

The most proximal V_H genes escape allelic exclusion in M54 transgenic B lymphocytes. (**a**) V_HQ52 and V_H7183 gene families of the mouse C57BL/6 strain. The family members were identified by annotation of the *Igh* sequences of the mouse genome database (http://mendel.imp.univie.ac.at/SEQUENCES/VH/). The V_H genes are numbered according to published nomenclature[11]. b, C57BL/6; p, pseudogenes. Our annotation identified previously unknown family members (n). Thick horizontal black bars indicate a large sequence duplication; genes with high sequence similarity are in the same color. The $V_H7183.b2$ gene segment of the C57BL/6 mouse corresponds to the V_H81X gene segment of the BALB/c strain. (**b,c**) Statistical analysis of V_H-DJ_H rearrangements involving different members of the V_HQ52 (**b**) and V_H7183 (**c**) gene families in wild-type and *Pax5*$^{-/-}$ pro–B cells, *Ik*$^{Pax5/+}$ pro–T cells and M54 transgenic IgMa B cells. V_H-DJ_H4 rearrangements of pro–B and pro–T cells and V_H-DJ_H3 rearrangements of M54 B cells were amplified by PCR, cloned, sequenced and assigned to the various family members. Data represent the percentage of rearrangements involving individual genes for each cell type. Total number of distinct rearrangements analyzed: wild-type pro–B cells, 76 (V_HQ52) and 123 (V_H7183); *Pax5*$^{-/-}$ pro–B cells, 61 (V_HQ52) and 83 (V_H7183); *Ik*$^{Pax5/+}$ pro–T cells, 73 (V_HQ52) and 81 (V_H7183); splenic M54 B cells, 71 (V_HQ52) and 79 (V_H7183).