Peripheral subnuclear positioning suppresses Tcrb recombination and segregates Tcrb alleles from RAG2

Elizabeth A. W. Chan, Grace Teng, Elizabeth Corbett, Kingshuk Roy Choudhury, Craig H. Bassing, David G. Schatz, and Michael S. Krangel

Departments of *Immunology,* 1 Radiology, and 2Biostatistics and Bioinformatics, Duke University Medical Center, Durham, NC 27710; 3Department of Immunobiology and 4Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520; and 5Department of Pathology and Laboratory Medicine, Children’s Hospital of Pennsylvania, Philadelphia, PA 19104

Edited by Anjana Rao, La Jolla Institute for Allergy and Immunology, La Jolla, CA, and approved October 22, 2013 (received for review June 7, 2013)

Allelic exclusion requires that the two alleles at antigen-receptor loci attempt to recombine variable (V), diversity (D), and joining (J) gene segments [V(D)J recombination] asynchronously in nuclei of developing lymphocytes. It previously was shown that T-cell receptor β (Tcrb) alleles frequently and stochastically associate with the nuclear lamina and pericentromeric heterochromatin in CD4+ CD8+ thymocytes. Moreover, rearranged alleles were under-represented at these locations. Here we used 3D immunofluorescence in situ hybridization to identify recently rearranged Tcrb alleles based on the accumulation of the DNA-repair protein 53BP1. We found that Tcrb alleles recombine asynchronously in double-negative thymocytes. Moreover, rearranged alleles were asynchronous in double-negative thymocytes and that V(D)J recombination is suppressed on peripheral as compared with central Tcrb alleles. Moreover, the recombination events that did take place at the nuclear periphery preferentially occurred on Tcrb alleles that were partially dissociated from the nuclear lamina. To understand better the mechanism by which V(D)J recombination is suppressed at the nuclear periphery, we evaluated the subnuclear distribution of recombination-activating gene 2 (RAG2) protein. We found that RAG2 abundance was reduced at the nuclear periphery. Moreover, 53BP1 was distributed differently from RNA polymerase II and histone H3K4 trimethylation. Our data suggest that the nuclear periphery suppresses V(D)J recombination, at least in part, by segregating Tcrb alleles from RAG proteins.

T-cell development / thymus

Antigen receptor variable (V), diversity (D), and joining (J) gene segments are assembled by V(D)J recombination in immature T and B lymphocytes to generate diverse repertoires of T-cell receptors (TCRs) and B-cell receptors (BCRs), respectively (1). V(D)J recombination is initiated by the recombination-activating gene (RAG) 1 and 2 proteins, which bind to and induce double-strand breaks (DSBs) at recombination signal sequences that flank V, D, and J segments. V(D)J recombination at antigen-receptor loci is regulated according to cell lineage and developmental stage (2). In addition, at some loci V (D)J recombination is regulated to enforce allelic exclusion, so that a complete antigen-receptor protein is produced by only one allele (3, 4). However, the mechanisms that establish allelic exclusion are poorly understood.

Among TCR loci, only the T-cell receptor β (Tcrb) locus is allelically excluded (5). Tcrb recombination occurs in CD4+ CD8− double-negative (DN) thymocytes and is ordered, beginning with DP− Jβ rearrangement, which can occur on both alleles. Allelic exclusion then is initiated by Vβ-to-DJβ rearrangement, which is thought to occur asynchronously, i.e., on one allele at a time. This asynchrony allows thymocytes time to test each allele for the creation of an ORF. TCRβ proteins are sensed by their assembly with pre-TCR and CD3 chains to create a pre-TCR signaling complex; pre-TCR signals then suppress further Tcrb recombination and promote thymocyte proliferation and differentiation to the CD4+ CD8+ double-positive (DP) stage (6).

Allelic exclusion is maintained in DP thymocytes in part by chromatin alterations, such as reduced Vβ germ-line transcription and histone acetylation, that reduce access of RAG1/2 proteins to Vβ gene segments (7). In addition, Tcrb alleles adopt a more extended, or decontracted, conformation in DP thymocytes, physically separating Vβ and DJβ segments (8). Loss of accessibility and locus decontraction both contribute to the maintenance of allelic exclusion, because Vβ and DJβ segments engineered to be accessible and proximal are capable of recombination in DP thymocytes (9, 10). However, because both Tcrb alleles appear to be accessible (11, 12) and contracted (8) before rearrangement in DN thymocytes, the mechanism by which the locus is biased to undergo asynchronous Vβ-to-DJβ recombination in DN thymocytes is unknown.

It has been suggested that subnuclear positioning can regulate V(D)J recombination at TCR and BCR loci. For example, association with pericentromeric heterochromatin (PCH) has been linked to the process of allelic exclusion. Igh loci were shown to associate with PCH monoallelically in roughly 70% of pre-B cells. Moreover, the recruited alleles were decontracted, suggesting that they had not undergone VH rearrangement (13). Tcrb alleles have been shown to associate with PCH in a regulated (8) or stochastic (14) fashion in different studies. Direct analysis of rearrangement status revealed that PCH-associated Tcrb alleles tend not to have undergone VH rearrangement (14).

The positioning of TCR and BCR alleles at the nuclear periphery also is thought to inhibit V(D)J recombination. Most Igh and Ijk alleles are located at the nuclear periphery in non-B lineage cells, whereas in pro-B cells they become more centrally located (15). This relocalization is thought to occur as a prelude

Eukaryotic genes are directed to distinct subnuclear compartments to regulate their activity. We show that different regions of the murine T-cell receptor β (Tcrb) locus interact independently with the nuclear lamina and that these interactions locally suppress the recombination of variable, diversity, and joining gene segments. This suppression is associated with the physical segregation of the locus from the recombination protein, recombination-activating gene 2. Allelically excluded recombination of antigen receptor genes promotes the development of lymphocytes that each express a single antigen receptor. We propose that interaction with the nuclear lamina contributes to allelic exclusion by reducing the frequency of recombination of Tcrb alleles.

Author contributions: E.A.W.C. and M.S.K. designed research; E.A.W.C. performed research; G.T., E.C., C.H.B., and D.G.S. contributed new reagents/analytic tools; E.A.W.C., K.R.C., C.H.B., and D.G.S. wrote the paper.

The authors declare no conflict of interest.

This article is PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: krang001@mc.duke.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1310846110/-/DCSupplemental.
to expression and V(D)J recombination. Tcrb alleles localize stochastically to the nuclear periphery in DN thymocytes, with most nuclei having either one or two associated alleles (14). Peripheral Tcrb alleles were less likely than more central alleles to have undergone Vβ-to-DJβ rearrangement (14), suggesting that association with the nuclear periphery may suppress recombination and contribute to allelic exclusion. However, this analysis tracked Tcrb alleles that already were rearranged, so it is possible that recombination occurs freely at the nuclear periphery, with rearranged alleles subsequently relocating away from this compartment.

Here we visualized recently rearranged Tcrb alleles based on the assembly of foci containing DNA repair factors, and we provide evidence that Tcrb alleles recombine asynchronously in DN thymocyte nuclei. We found that peripheral Tcrb alleles are marked by repair foci less frequently than central Tcrb alleles. Moreover, the DNA repair foci that were detected on peripheral Tcrb alleles tended to occur on alleles that were partially dissociated from the nuclear lamina. To understand better why DNA repair foci are underrepresented on peripheral Tcrb alleles, we evaluated the location of RAG2 relative to Tcrb alleles and the nuclear lamina. We found that peripheral Tcrb alleles reside in a region of relatively low RAG2. This result suggests that the nuclear periphery helps to suppress V(D)J recombination by segregating Tcrb alleles from functional RAG proteins.

Results

**Tcrb Alleles Recombine Monoallelically in DN Thymocytes.** We evaluated the frequency of recent RAG-dependent DSBs in DN thymocytes by tracking DNA-repair foci using an antibody specific for the repair protein 53BP1. 53BP1 becomes phosphorylated by ataxia-telangiectasia mutated (ATM) and is recruited to DSBs in sufficient amounts to form visible foci, indicative of ongoing or recent DNA repair (16). 53BP1 foci form within minutes of a DSB and may remain at DSB sites for several hours after their repair (17). 53BP1 also has been implicated in V(D)J recombination and class-switch recombination, functioning to protect DNA ends and to support long-range DNA interactions (18-20).

To analyze 53BP1 foci immediately ex vivo, we isolated DN thymocytes from linker for activation of T cells-negative (Lat−) mice. These thymocytes are unable to signal through pre-TCR components and therefore cannot progress beyond the CD44−CD25+ DN3 stage of development (21). As a control, we also isolated DN thymocytes from RAG2−/− mice, because thymocytes are similarly blocked in development at the DN3 stage but should accumulate only random DSBs that are unrelated to V(D)J recombination. We excluded from analysis those nuclei considered to be in the process of apoptosis. Nuclei in this category (<10% of all nuclei) contained intense and broadly distributed 53BP1 staining or more than six 53BP1 foci. We chose six foci as a cutoff because that is the maximum number of Tcrb, Terg, and Tcrg alleles that potentially could undergo recombination in DN thymocytes. Among 500 Lat− nuclei analyzed, 23% contained a single 53BP1 focus, 5% contained two foci, less than 1% contained three or four foci, and none contained five or six foci (Fig. 1A). Similar examination of 500 Rag2−/− DN nuclei identified 12% with one 53BP1 focus, 0.2% with two foci, and none with three to six foci. From these results we conclude that the majority of 53BP1 foci detected in Lat−/− nuclei reflect DSBs resulting from V(D)J recombination. Given the overall frequency of DSBs and the rarity of nuclei with more than two DSBs, we conclude that V(D)J recombination occurs infrequently at TCR loci in DN3 thymocytes.

![Fig. 1. Tcrb alleles recombine infrequently and asynchronously in Lat−/− DN thymocytes.](Image)

To identify RAG-dependent DSBs specifically at Tcrb alleles, we used DNA immuno-FISH to visualize 53BP1 and Tcrb alleles simultaneously (Fig. 1B). We measured the distance between the center of each 53BP1 focus and the center of the nearest Tcrb focus in 500 Lat−/− and 500 Rag2−/− DN thymocytes. We detected a discrete population of DSBs within 1 μm of a Tcrb focus in Lat−/− nuclei but did not detect a similar population in Rag2−/− nuclei. Based on this finding, we concluded that 53BP1 foci within 1 μm of a Tcrb allele identify Tcrb alleles that recently have undergone V(D)J recombination. We note that 53BP1 foci farther than 1 μm from a Tcrb focus were twice as frequent in Lat−/− as in Rag2−/− nuclei (118 vs. 61 foci). We presume that this increase reflects recent RAG-mediated DSBs at Terg and Tcrg loci in Lat−/− nuclei.

It has been assumed that Tcrb alleles may undergo synchronous Dβ-to-Jβ recombination followed by asynchronous Vγ-Jγ recombination during the initiation phase of allelic exclusion in DN thymocytes. However, direct evidence addressing either of these points has been lacking. We found that ~90% of Lat−/− DN thymocytes contained no 53BP1+ Tcrb alleles, 9.4% contained a single 53BP1+ Tcrb allele, and only 0.4% contained two 53BP1+ Tcrb alleles (Fig. 1C). A recent study demonstrated 53BP1+ Tcrb alleles in a somewhat smaller fraction (4%) of DN2/3 thymocytes of WT mice (22). In contrast to the low frequency of nuclei with two 53BP1+ Tcrb alleles in DN thymocytes of Lat−/− mice, we readily detected nuclei with two 53BP1+ Tcrb alleles in DN thymocytes of mice that are impaired in coding joint formation because of Artemis deficiency (Fig. 1D). In this case, 36.4% of DN thymocyte nuclei contained one 53BP1+ Tcrb allele, and only 0.4% contained two 53BP1+ Tcrb alleles. (A) (Upper) Confocal immunofluorescence microscopy showing single z-slices of Lat−/− DN thymocyte nuclei containing one or two 53BP1 foci. (Scale bars, 1 μm.) (Lower) Distributions of Rag2−/− and Lat−/− DN thymocyte nuclei containing one to six 53BP1 foci. (B) (Upper) Confocal immuno-FISH microscopy showing single z-slices of Lat−/− DN thymocyte nuclei containing 53BP1 foci at or distant from Tcrb foci. (Scale bars, 1 μm.) (Lower) Distance of 53BP1 foci from the nearest Tcrb allele in 500 Rag2−/− and 500 Lat−/− DN thymocyte nuclei. Tcrb alleles were identified using the Vβ probe. The horizontal line at 1 μm separates presumed 53BP1 foci from other 53BP1 foci. (C) Distribution of nuclei containing one or two 53BP1+ Tcrb alleles in 500 Lat−/− DN thymocytes. (D) Distribution of nuclei containing one or two 53BP1+ Tcrb alleles in 173 Artemis−/− DN thymocytes. Data for Rag2−/−, Lat−/−, and Artemis−/− DN thymocytes were each compiled from two independent experiments.
allele, and 9.3% contained two 53BP1+ Tcrb alleles. The rare Lat−/− nuclei containing two 53BP1+ Tcrb alleles could result from the simultaneous rearrangement of both alleles; however, because 53BP1 foci can remain at the site of a DSB for several hours (17, 23), they also could be explained by rearrangement events occurring hours apart. We conclude that Tcrb recombination occurs infrequently in Lat−/− DN thymocytes. Moreover, because the detected 53BP1 foci likely represent a mixture of DJβ-to-Jβ and Vβ-to-DJβ recombination events, we conclude that both steps of Tcrb recombination are likely to occur asynchronously on Tcrb alleles in DN thymocytes.

Reduced Tcrb Recombination at the Nuclear Periphery. The nuclear periphery is composed of inner and outer nuclear membranes, nuclear pore complexes, and the nuclear lamina (24). Approximately 40% of the chromatin in Drosophila, mouse, and human nuclei interact with the nuclear lamina. Regions of interacting chromatin, termed “lamin-associated domains” (LADs) (25), can span 0.1–10 Mb. LADs typically contain regions of inactive chromatin and have sharp boundaries that are demarcated by CpG islands, promoters, and CCCTC-binding factor (CTCF) sites (26–28). Although most LADs are similar across cell types, developmentally regulated genes have been shown to be relocated toward or away from the nuclear lamina in a manner that generally correlates with their activation state (29–32). Additionally, when certain active genes have been forcibly repositioned to the nuclear lamina, their transcription was reduced (33–36). Therefore, the nuclear lamina is thought to represent a repressive subnuclear compartment.

Previous work showed that ~45% of DN thymocyte nuclei contain two lamin-associated Tcrb alleles, and an equal number contain one lamin-associated and one free allele. Within the latter subset, fully rearranged Tcrb alleles were substantially underrepresented at the lamina (14). To address more directly whether association with the nuclear lamina may suppress ongoing Tcrb recombination, we assessed the locations of recently rearranged Tcrb alleles in Lat−/− nuclei containing a single 53BP1+ Tcrb allele and a single Tcrb allele in contact with the nuclear lamina. We scored contact when two adjacent pixels were positive for both laminB1 staining and Tcrb hybridization. When association with the lamina was assessed using a probe hybridizing with the Vβ end of the Tcrb locus (Fig. 2A), 53BP1+ foci were not significantly underrepresented on WT Tcrb alleles in Lat−/− thymocytes (Fig. 2B).

The DJβ-to-Jβ and Vβ-to-DJβ steps of Tcrb recombination could be differentially affected by Vβ-end contact with the lamina. Therefore, a specific effect on one step of recombination could have been masked in the above experiment. To distinguish 53BP1 foci arising from the two steps of recombination, we used gene-targeted mice carrying modified Tcrb alleles that can undergo only DJβ-to-Jβ or only Vβ-to-DJβ recombination events (Fig. 2D). The M4 Tcrb allele has a mutation in the 5′ D1J1 RSS and lacks the D2J12β cluster, and thus it can undergo only DJβ-to-Jβ recombination (37). The DJβ allele has a pre-rearranged DJ11 gene segment and lacks the D2J12β cluster and thus can undergo only Vβ-to-DJ1β recombination (38). The ω allele serves as a useful control because it lacks the D2J12β cluster but has an unmanipulated DJ1J1β cluster and thus can undergo both DJβ-to-Jβ and Vβ-to-DJ1β recombination (37). All modified alleles were crossed onto a Lat− background to allow the direct harvest and analysis of DN thymocytes immediately ex vivo. Consistent with the loss of half of the D1 and Jβ segments, the frequency of 53BP1+ ω Tcrb alleles was roughly half that of WT alleles (Fig. 2D). The frequencies of 53BP1+ M4 and DJβ alleles were only slightly lower (Fig. 2D). We note that 53BP1 foci reflecting Vβ-to-DJ1β recombination may be more frequent in DJβ/DJβ Lat−/− than in ω/ω Lat−/− nuclei because Vβ-to-DJβ recombination was found to occur more efficiently on alleles that already have undergone DJβ-to-Jβ recombination (38).

Similar to the results obtained with Lat−/− thymocytes, we found no evidence that 53BP1 foci were underrepresented on Tcrb alleles anchored to the nuclear lamina by the Vβ end in either ω/ω Lat−/− or M4/M4 Lat−/− thymocytes (Fig. 2B). However, DJβ/DJβ Lat−/− thymocytes behaved differently, because only 26% of 53BP1 foci occurred on Tcrb alleles in contact with the nuclear lamina, and 74% occurred on free alleles. This result implies that Vβ-end association with the nuclear lamina suppresses the Vβ-to-DJβ but not the DJβ-to-Jβ step of Tcrb recombination.

Given the selective impact of Vβ-end association on Vβ-to-DJβ recombination, we hypothesized that recombination could be differentially influenced depending on the nature of Tcrb locus contact with the nuclear lamina. Therefore, we also scored Tcrb–lamin colocalization using a probe that detects the Cβ end of the Tcrb locus (Fig. 2A and C). We observed substantially reduced frequencies of 53BP1+ foci on Cβ end-associated Tcrb alleles in Lat−/−, M4/M4 Lat−/−, and DJβ/DJβ Lat−/− thymocytes (Fig. 2C). We also observed a similar trend in ω/ω Lat−/− thymocytes, al-

Fig. 2. Tcrb recombination is suppressed at the nuclear periphery. (A) Diagram of modified Tcrb alleles and location of Vβ-end and Cβ-end DNA probes. WT and ω alleles can undergo DJβ-to-Jβ and Vβ-to-DJβ recombination, whereas M4 alleles can undergo only DJβ-to-Jβ and DJβ alleles can undergo only Vβ-to-DJβ recombination. Frequencies of 53BP1 foci were calculated from 500 WT, 1,100 ω, 1,525 M4, and 394 DJβ nuclei on two to four slides per genotype. (B) Frequencies of lamin-associated and free 53BP1+ Tcrb alleles in Lat−/− DN thymocyte nuclei having a single 53BP1+ Tcrb allele and monoallelic Tcrb association with the nuclear lamina. Tcrb association with the lamina was scored using a Vβ-end probe on 63 WT, 56 ω, 61 M4, and 54 DJβ nuclei (five to eight slides per genotype). (C) Tcrb association with the lamina was scored using a Cβ-end probe on 70 WT, 52 ω, 51 M4, and 58 DJβ nuclei (four to eight slides per genotype). Fisher’s exact two-tailed contingency tables were used to compare frequencies of lamin-associated 53BP1+ Tcrb alleles to the frequency of total lamin-associated Tcrb alleles (50%). *P < 0.05; **P < 0.01; ns, not significant.
though the result fell short of statistical significance (Fig. 2C). These results imply that Cβ-end association with the nuclear lamina suppresses both the Dβ2-to-Jγ and Vβ-to-DJγ steps of Tcrb recombination. We conclude that the Vβ and Cβ ends of the locus may associate with the nuclear lamina independently. Tethering by the Vβ end selectively inhibits Vβ-to-DJγ recombination because the distant Dβ2 and Jγ segments may remain free of the nuclear lamina and available for recombination. In contrast, tethering by the Cγ end inhibits both Dβ2-to-Jγ and Vβ-to-

**Distinct Conformations of Tcrb Alleles at the Nuclear Lamina.** Independent interactions of the Vβ and Cβ ends suggest that lamin-associated Tcrb alleles may adopt distinct conformations relative to the nuclear lamina that may be suppressive or permissive for recombination. To assess this possibility more directly, we evaluated the orientation of lamin-associated 53BP1− and total Tcrb alleles by deconvolving 3D images and measuring the distances between the nearest point on the inner surface of the nuclear lamina and the centers of Tcrb and 53BP1 foci.

We first analyzed the conformations of peripheral Tcrb alleles by comparing the locations of the Vβ and Cβ ends of individual Tcrb alleles relative to the nuclear lamina in Lat−/− thymocytes (Fig. 3A, Left). We then calculated the extent to which one end of the Tcrb locus was more embedded in the lamina or more nucleoplasmic than the other end (Fig. 3B). Alleles anchored to the lamina via the Vβ end displayed a spectrum of conformations. On most alleles the Cβ end was displaced less than 100 nm relative to the Vβ end (Fig. 3C, Left). However, on some alleles the Cβ end was substantially more nucleoplasmic than the Vβ end. Similar conclusions were reached for Vβ-end displacement when the Cβ end was positioned at the lamina (Fig. 3D, Left). These data suggest that the entire lengths of most peripheral Tcrb loci interact with the lamina, whereas on some alleles one end may be free of the lamina. These observations held true for the modified Tcrb alleles as well, showing that none of the modifications substantially influence the conformation of the Tcrb locus relative to the lamina.

We then analyzed the displacement of 53BP1 foci relative to the lamin-associated Vβ end of the Tcrb locus (Fig. 3A, Center and Fig. 3C, Right). 53BP1 foci almost always were farther from the lamina than the lamin-associated Vβ end, being on average 150–230 nm more nucleoplasmic in the different genotypes. This observation suggests that, although V(D)J recombination is suppressed by the nuclear lamina, Vβ-to-DJγ and Dβ2-to-Jγ recombination may occur on Tcrb alleles that have partially dissociated on the Cγ end.

Similarly, 53BP1 foci were farther from the lamina than the lamin-associated Cγ end of the Tcrb locus, being on average 87–126 nm more nucleoplasmic in the different genotypes (Fig. 3A, Right and Fig. 3D, Right). That 53BP1 foci tended to be less well separated from the Cγ end than from the Vβ end makes sense, because the Cγ-end probe is centered ~140 kb downstream of the Dβ2 and Jγ segments, whereas the Vβ-end probe is centered ~325 kb upstream of the center of the Vγ array (Fig. 2A). We conclude that the full lengths of most Tcrb alleles are in contact with the nuclear lamina but that V(D)J recombination occurs preferentially on the subset of peripheral alleles that have partially dissociated from the lamina.

**RAG2 Levels Are Reduced at the Nuclear Periphery.** Contact of the Tcrb locus with the nuclear lamina could function to suppress V(D)J recombination by inhibiting transcription and accessibility of Tcrb alleles. However, prior work indicated that Vγ segments are transcribed biallelically in all DN thymocytes (12). Another possibility is that peripheral Tcrb loci might be inhibited from undergoing V(D)J recombination because of their segregation from RAG proteins. In this regard, a recent publication demonstrated that the histone H3 lysine 4 trimethylation (H3K4me3) modification is sequestered to the nuclear interior (39). Because RAG2
contains a plant homeodomain (PHD) finger that interacts with H3K4me3 (40-42), functional RAG proteins may be similarly sequestered. Therefore we investigated the subnuclear distribution of RAG2 relative to the nuclear lamina.

An anti-RAG2 monoclonal antibody (43) detected punctate staining of variable intensity in about 70% of Lat−/− nuclei but not in control Rag2−/− nuclei (Fig. 4A). We also co-stained with anti-Ki67, because this protein marks cycling cells (44), and RAG2 is degraded in a cell-cycle-dependent fashion (45). As expected, RAG2 staining was generally low in the Ki67+ subset (∼15%) of Lat−/− nuclei but was readily detected in most Ki67low nondividing nuclei (Fig. 4D). Within Rag2−/− nuclei, RAG2 staining was excluded from areas of heterochromatin, defined by concentrated DAPI staining (Fig. 4B). To provide further evidence for staining specificity, we examined additional populations of T and B cells. As expected from the known expression characteristics of RAG2 protein, staining with anti-RAG2 was detected in DP thymocytes but not in more mature CD4+CD8− thymocytes (Fig. S1A). Staining also was observed in pro-B cells but not in mature peripheral B cells (Fig. S1B). The punctate staining distributions in DP thymocyte and pro-B-cell nuclei were similar to those observed in DN thymocyte nuclei.

To evaluate the RAG2 distribution relative to the nuclear periphery, we deconvolved 3D images and obtained fluorescence intensity traces across the nuclear diameter of individual thymocytes. These traces revealed peaks of RAG2 staining between laminB1 peaks in Lat−/− but not Rag2−/− control nuclei (Fig. 4C). We then summed fluorescence intensity plots from 40 nuclear edges per experiment to observe average distributions of RAG2 in Lat−/− and Rag2−/− nuclei relative to peak lamin intensity (Fig. 4D). These results revealed that RAG2 staining does not reach maximum intensity until nearly 1 μm from peak lamin staining, suggesting that RAG2 is excluded from the nuclear periphery.

Segregation of Peripheral Tcrb Alleles from RAG2. To understand the distribution of Tcrb alleles relative to the nuclear periphery and RAG2, we obtained traces measuring the shortest distance between peak intensities of Tcrb foci and the nuclear lamina. Tcrb alleles, on average, distributed very close to the nuclear lamina (Fig. 5A). In fact, they were as close to the lamina as the most peripheral DNA, defined by DAPI staining. In this position, Tcrb alleles were segregated from the highest concentrations of RAG2. In contrast, Tcra/Tcra alleles, which are not allelically excluded, were distributed more centrally in the nucleus in an environment characterized by high levels of RAG2 (Fig. 5A).

To analyze further the spatial relationships between Tcrb alleles and RAG2, we simultaneously visualized Tcrb alleles and RAG2 protein in individual DN thymocyte nuclei (Fig. 5B). The great majority of Tcrb alleles embedded within the nuclear lamina did not colocalize with RAG2 foci (Fig. 5B, i and Fig. 5C, right bar). Moreover, Tcrb alleles in contact with the nuclear lamina (Fig. 5B, ii−v and Fig. 5C, center bar) colocalized with RAG2 foci less frequently than did free Tcrb alleles (Fig. 5B, vii−ivii and Fig. 5C, left bar). Taken together, these data suggest that positioning at the nuclear periphery reduces the frequency with which Tcrb alleles interact with RAG2.

The Subnuclear Distribution of RAG2 Is Distinct from That of RNA Polymerase II and H3K4me3. The experiments described above raise the question of what determines the subnuclear distribution of RAG2 proteins. We characterized the subnuclear distributions of H3K4me3 and RNA polymerase II (PolII) in Lat−/− DN thymocyte nuclei by staining with specific antibodies and comparing fluorescence intensity plots with those for the nuclear lamina, RAG2, and DAPI staining (Fig. 6A). Antibodies used for H3K4me3 and PolII staining were judged to be specific based on blocking with the respective immunogenic peptides (Fig. 6B and E).

Neither H3K4me3 nor PolII fluorescence intensity extended as close to the nuclear lamina as total DNA, in agreement with the idea that the nuclear periphery generally is transcriptionally repressive. However, H3K4me3 and PolII staining appeared to peak slightly closer to the nuclear lamina than RAG2 staining.
sugesting that the RAG2 distribution may not be defined by
either factor.
To evaluate better the subnuclear relationship between RAG2 and PolII, we simultaneously visualized both proteins in individual thymocyte nuclei (Fig. 6B). The distributions of the two proteins overlapped only partially, with minimal correlation between the two fluorescence intensity signals in individual thymocyte nuclei (Fig. 6C, Left) and as averaged over many nuclei in several independent experiments (Fig. 6D). Hence RAG2 protein does not localize primarily to transcription factories.

Technical limitations prevented us from directly comparing the RAG2 distribution with that of H3K4me3. However, we were able to coinvalize H3K4me3 and PolII (Fig. 6E). We observed substantially overlapping and highly correlated distributions (Fig. 6C, Right, and D), consistent with the localization of H3K4me3 near the promoters of actively transcribed genes (46). Thus, although RAG2 has a PHD domain that binds H3K4me3 (40, 41), and chromatin-bound RAG2 colocalizes with H3K4me3 genome wide (47), we infer that the distribution of total RAG2 protein in DN thymocyte nuclei is unlikely to correspond well to the distribution of H3K4me3.

To address the role of H3K4me3 in determining the subnuclear distribution of RAG2 protein independently, we analyzed the RAG2 distribution in DN thymocytes of mice expressing a truncated RAG2 protein that lacks its C-terminal region, including the PHD domain (48). Comparison of Rag<sup>−/−</sup> Lat<sup>+/+</sup> and Lat<sup>−/−</sup> DN thymocytes revealed indistinguishable distributions of RAG2 protein relative to the nuclear lamina in the two cell populations (Fig. 7). This result indicates that binding to H3K4me3 does not limit RAG2 protein from the nuclear periphery and reinforces the notion that binding to H3K4me3 does not dictate the subnuclear distribution of total RAG2 protein.

**Tcrb Alleles Predominantly Colocalize with PolII.** Because the nuclear lamina is thought to be repressive for transcription, the inhibition of V(D)J recombination on peripheral Tcrb alleles could reflect, in part, segregation from RNA PolII and reduced transcription and accessibility to RAG proteins. To address this point, we simultaneously visualized Tcrb alleles and PolII in DN thymocyte nuclei (Fig. 8A). Almost all Tcrb alleles colocalized with PolII signal, regardless of their peripheral localization (Fig. 8A and B). Notably, Tcrb alleles almost always were positioned at the edge of regions of PolII staining, and we often observed finger-like projections of PolII extending to Tcrb alleles at the nuclear lamina (Fig. 8A, i and vii). Moreover, in most nuclei, both Tcrb alleles were in contact with PolII (Fig. 8A, i–iv and vi). Thus, our results are consistent with previous work demonstrating that V<sub>β</sub> gene segments are transcribed biaxially in DN thymocyte nuclei (12). We conclude that the peripheral location of many Tcrb alleles may limit recombination primarily by impacting the supply of RAG2 to accessible Tcrb alleles.

**Discussion**

Previously we showed that Tcrb alleles stochastically localize to the nuclear periphery and that peripheral Tcrb alleles are less likely to have undergone V<sub>β</sub>-to-DJ<sub>β</sub> recombination. Here we found a reduction of 53BP1<sup>+</sup> Tcrb alleles at the nuclear periphery, offering strong support for the notion that ongoing Tcrb recombination is suppressed by interaction of Tcrb alleles with the nuclear lamina (Fig. 8A, i and vii). Moreover, in most nuclei, both Tcrb alleles were in contact with PolII (Fig. 8A, i–iv and vi). Thus, our results are consistent with previous work demonstrating that V<sub>β</sub> gene segments are transcribed biaxially in DN thymocyte nuclei (12). We conclude that the peripheral location of many Tcrb alleles may limit recombination primarily by impacting the supply of RAG2 to accessible Tcrb alleles.
Our conclusions rest on the assumption that repair of RAG-induced DSBs occurs in the same location in which they were generated. DSB repair in yeast has been shown to involve movement and clustering of DNA repair foci (49). Studies of DSB repair following radiation-induced damage in mammalian nuclei have documented mobility and clustering of repair foci, as in yeast (50), or minimal mobility that is no greater than that of undamaged chromatin (51, 52). Notably, positional stability also was documented for single enzymatically induced DSBs (53, 54). This result also is consistent with studies indicating that chromosomal translocations tend to occur between loci that already are in spatial proximity at the time of DNA damage (53-56). Thus, we think it likely that the Tcrb-proximal 53BP1 foci we observe mark the subnuclear sites of Tcrb recombination events.

RAG-induced DSBs activate the ATM kinase, which aids in efficient DSB repair (57). ATM recently was shown to help enforce allelic exclusion at Igk loci by downregulating Rag1 and Rag2 transcription in response to a RAG-induced DSB (58). Transient suppression of recombination by this mechanism could guarantee asynchronous recombination by preventing recombination on the second allele while the first allele is being repaired and tested for functionality. Should such a mechanism function in DN thymocytes, stringent regulation of Tcrb recombination would predict uniformly monoallelic Tcrb 53BP1 foci in Artemis-deficient thymocytes, because these thymocytes cannot repair RAG-induced coding ends and would signal continuously to suppress Tcrb recombination on the other allele. However, we found that nearly 10% of Artemis-deficient DN thymocyte nuclei contained two 53BP1+ Tcrb alleles. Moreover, if such a mechanism were active, it also should prevent concurrent rearrangement events at different TCR loci in DN thymocytes. However, nuclei with 53BP1 foci at two or three TCR loci do not appear to be underrepresented in WT DN thymocytes (22). We conclude that signaling downstream of an unrepaired Tcrb DSB is insufficient to ensure monoallelic RAG activity, perhaps because RAG protein expression cannot be downregulated rapidly enough to guarantee this outcome. It remains unclear whether this insufficiency applies selectively to Dp+ to-Djβ recombination, which likely predominates in Artemis-deficient DN thymocyte nuclei, or applies to Vp+ to-Djβ recombination as well.

Our conformational analysis of the Tcrb locus suggested that on most lamin-associated Tcrb alleles both the Vp and Cγ ends of the locus were associated with the nuclear lamina. However, on 53BP1+ Tcrb alleles, the 53BP1 focus generally was farther from the nuclear lamina than the lamin-associated Vp end and Cγ end of the locus. Our interpretation was that one end of the locus had dissociated from the lamina and therefore was permissive for recombination. However, an alternate interpretation of our results would be that 53BP1+ Tcrb alleles are anchored to the lamina via both ends of the locus, with 53BP1 accumulating on central Tcrb-locus DNA that loops away from the periphery. We could not analyze this possibility directly, because technical limitations prevented us from performing four-color 3D immuno-FISH analysis in which the nuclear lamina, 53BP1, the Vp end, and the Cγ end of the Tcrb locus could be labeled...
recombination is inhibited on alleles anchored to the
locus is positioned at the nuclear lamina. A recent
Rag2 and alleles in DN thymocyte nuclei
alleles that colocalize with PolII. Data were
PNAS PLUS
Published online November 11, 2013
alleles are
Igh
and 186 alleles in
Cyp3a
recombination by regulating locus transcription and
segments in DN thymo-
Igh
Tcrb
Tcrb
segments are dissociated from the lamina.
ive of subnuclear localization,
Nearly all
nuclei to allow evaluation of relative
DN thymocytes;

simultaneously and distinctly. However, our observation that D
β
to-Jβ recombination is inhibited on alleles anchored to the
lamina via their Cβ but not their Vβ end argues, at a minimum,
that these must represent two conformationally distinct subsets
of alleles.

An important unresolved question is the mechanism by which
the TcRb locus is positioned at the nuclear lamina. A recent
publication identified specific DNA sequences in the lamin-
associated Igh and Cyp3a genes that could recruit heterologous
genes to the lamina in NIH 3T3 cells (36). These fragments
contain multiple binding motifs for the transcriptional repressor
cKrox. Additionally, both cKrox and its interaction partner
HDAC3 were shown to be significant mediators of Igh and Cyp3a
lamin association. CTCF also has been implicated in perinuclear
positioning of a subtelomeric element (59) and of the inactive
human cystic fibrosis transmembrane conductance regulator
gene (60). More recently, high AT content was shown to be
a defining feature of constitutive LADs (61). It will be important
to dissect the TcRb locus with the goal of identifying peripheral
targeting elements and transacting factors in future experiments.

Although we found that the majority of TcRb alleles are
present at the nuclear periphery in an environment characterized
by reduced levels of Rag2, ChIP analysis has demonstrated
abundant Rag2 binding to Dβ and Jβ segments in DN thymo-
ocytes (47). Although these results seem contradictory, it is
important to consider that ChIP provides only a population analysis
that cannot discriminate heterogeneity of Rag2 binding on
different alleles. We suggest that the Rag2 ChIP signals reflect
Rag2 binding on the subset of TcRb alleles that are free of the
nuclear lamina and the subset of peripheral TcRb alleles whose
Dβ and Jβ segments are dissociated from the lamina.

Although we have documented reduced Rag2 levels at the
nuclear periphery, we cannot formally rule out the possibility
that the nuclear periphery also may inhibit TcRb recombination
by other mechanisms. For example, the nuclear periphery could
exclude other components of the V(D)J recombination ma-
chinery, including Rag1 and DSB repair factors. Moreover,
because the nuclear periphery is generally suppressive for gene
expression, it remains possible that the nuclear periphery sup-
presses TcRb recombination by regulating locus transcription and
accessibility. One argument against this possibility was a study in
which a knockin reporter of Vβ8.2 expression was shown to be
biallelically transcribed in all DN thymocytes (12). However,
the possibility that Vβ8.2 has atypical expression characteristics or
that its expression was perturbed by introduction of the reporter
could not be formally excluded. Consistent with the notion of
biallelic transcription irrespective of subnuclear localization,
we detected PolII at 80–90% of lamin-associated and free
TcRb alleles in DN thymocyte nuclei. However, colocalization
with PolII does not indicate transcription. Thus, in future
studies it will be important to evaluate by RNA-FISH whether
germ-line transcription is equivalent on lamin-associated and
free TcRb alleles.

The molecular basis for the Rag2 distribution in DN thymo-
cyte nuclei remains unknown. Rag2 is known to interact
with the active histone modification H3K4me3 (40, 41), and
ChIP-sequencing analysis has revealed very high concordance
between Rag2 binding and H3K4me3 islands genome wide
(47). Although we could not compare the subnuclear distribu-
tions of Rag2 and H3K4me3 directly, our data do argue that
the overall Rag2 distribution is not defined by that of H3K4me3.
This apparent discrepancy may reflect the fact that the pop-
ulation view afforded by ChIP-sequencing overlooks the
dynamic nature of the spatial relationship between Rag2 and
transcription factories. We suggest that DN thymocyte nuclei
may contain both chromatin-bound and -free pools of Rag2.
We further suggest that Rag2 may move in and out of trans-
scription factories [which themselves appear to be quite dynamic
(62)], interacting with only a subset of actively transcribed genes
at any moment in individual nuclei. Consistent with the idea that
most nuclear Rag2 is either weakly associated or unassociated
with chromatin, Rag2 (but not PolII) staining was greatly
diminished when cells were briefly incubated with a standard
Triton X-100 wash buffer before fixation (Fig. S2). We contend
that the subnuclear distribution of this pool of Rag2 is highly

![Fig. 7. The RAG2 PHD domain is not required for Rag2 localization to the nuclear interior. Normalized distributions of Rag2 and laminB1 fluorescence intensity across Lat−/−, Rag2−/−, and Rag2−/− DN thymocyte nuclei. The Rag2 (red) and laminB1 (black) fluorescence intensity plots for Lat−/− and its corresponding Rag2+/− staining are from Fig. 4D. Slides containing Rag2+/− Lat−/− nuclei were stained and imaged together with an additional set of slides containing Rag2−/− nuclei to allow evaluation of relative fluorescence intensities (Rag2, blue; laminB1, gray). Traces were aligned and normalized as in Fig. 4D. Data were collected for 40 nuclear edges per experiment. Each trace represents the mean ± SEM of four or five independent experiments.](image_url)

![Fig. 8. Nearly all TcRb alleles colocalize with PolII. (A) Confocal immuno-FISH microscopy showing the relative locations of TcRb, PolII, and laminB1 in single z-slice images of Lat−/− DN thymocyte nuclei. TcRb alleles were identified by hybridization with the Cβ-end probe. (Scale bars, 1 μm.) (B) Frequencies of lamin-associated and free TcRb alleles that colocalize with PolII. Data were collected from 160 alleles in Rag2−/− and 186 alleles in Lat−/− DN thymocytes; ns, not significant using Fisher’s exact two-tailed contingency tables.](image_url)
relevant for the regulation of V(D)J recombination, because this pool would serve as the local source of RAG2 that becomes functionally associated with antigen-receptor loci. Additional studies clearly will be required to provide greater understanding of the assembly, subnuclear dynamics, and functional characteristics of recombinase foci in nuclei of developing lymphocytes.

Materials and Methods

Mice. Rag2−/− (63), Lat−/− (21), α (37), M4 (37), DjI (38), Artemis−/− (64), Rag2−/− (48), and pMT (65) mice were described previously. All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee.

Cell Collection. DN thymocytes were obtained directly from 2- to 3-week-old mice as previously described (14). DP and CD4+CD8− thymocytes were sorted from 2- to 3-week-old C57BL/6 or 129 mice using FITC-conjugated anti-CD4 (GK1.5) and Pacific Blue-conjugated anti-CD8α (53-6-7). Mature splenic B cells were obtained by sorting using Fc-block, anti-CD19 BIO (6D5) plus FITC-conjugated streptavidin, and Pacific Blue-conjugated anti-B220 (RA3-6B2). Pro-B cells were sorted from 4- to 6-week-old pMT mice using FITC-conjugated anti-CD43 (511) and Pacific Blue-conjugated anti-B220 (RA3-6B2). All antibodies were obtained from BioLegend.

FISH Probes and Antibodies. BAC clones RP23-75PS (at the Vj3 end of the Tcrb locus) and RP23-45TD7 (at the Cj3 end of the Tcra locus) were used as DNA probes. BAC clones were labeled using either a digoxigenin or biotin nick-translation kit (Roche). Foci were visualized with either FITC-conjugated anti-digoxigenin (200-092-211; Jackson Immunoresearch Laboratories) or Cy3-conjugated donkey anti-rabbit IgG (711-165-152; Jackson Immunoresearch Laboratories). The nuclear lamina was visualized using a polyclonal goat anti-lamin B1 antibody (sc-6217; Santa Cruz Biotechnology) and Cy5-conjugated anti-goat antibodies (705-605-003; Jackson Immunoresearch Laboratories). 53BP1 was visualized using a polyclonal rabbit anti-53BP1 antibody (NB 100-304; Novus Biologicals) and FITC-conjugated donkey anti-rabbit IgG (sc-2090; Santa Cruz Biotechnology). Ki67 was visualized using a polyclonal mouse anti-Ki67 antibody (556003; BD Pharmingen) and FITC-conjugated donkey anti-mouse IgG (715-095-150; Jackson Immunoresearch Laboratories). RAG2 was visualized using monoclonal rabbit anti-RAG2 antibody no. 39 (43) and Cy3-conjugated goat anti-rabbit IgG F(ab′)2 antibody (111-166-047; Jackson Immunoresearch Laboratories). Pol II was visualized using a monoclonal mouse anti-Pol II antibody (NB200-598; Novus Biologicals) and FITC-conjugated donkey anti-mouse IgG (715-545-151; Jackson Immunoresearch Laboratories). H3K4me3 was visualized using a polyclonal rabbit anti-H3K4me3 antibody (ab8580; Abcam) and either Cy3-conjugated donkey anti-rabbit IgG (711-165-152; Jackson Immunoresearch Laboratories) or FITC-conjugated donkey anti-rabbit IgG (711-095-152; Jackson Immunoresearch Laboratories).

3D DNA Immuno-FISH and Confocal Imaging. Methods for cell fixation and immuno-FISH were as described (14) except that slides were denatured at 77.8 °C before hybridization and were washed twice in 50% (vol/vol) formamide at 40 °C and three times in 0.2x SSC at 60 °C. Each slide received 0.5-1 µg of primary antibody and 3-5 µg of conjugated secondary antibody in 0.4 mL of 4% (wt/vol) BSA and 2x SSC. The specificity of Pol II and H3K4me3 staining was demonstrated by preincubation of primary antibody for each slide with 8 µg of Pol II (ab18488; Abcam) and H3K4me3 (ab1342; Abcam) peptide-immunogen, respectively, for 30 min at 23 °C before staining of nuclei. Slides were imaged on a Leica SP5 confocal microscope. Optical sections separated by 0.12 µm were collected, and only cells with intact laminB1 signals were analyzed. Nucleoplasmic proteins were extracted from cells by incubation of slides as previously described (66), except that cells were treated with 0.5% (vol/vol) Triton X-100, 20 mM Hepes (pH 7.9), 3 mM MgCl2, and 300 mM sucrose containing 0-100 mM NaCl for only 1 min. Slides then were processed for 3D immunofluorescence as described above.

Colocalization Analysis. All colocalization experiments were performed using ImageJ software. Images initially were passed through a Kalman stack colocalization plug-in. Images initially were processed in MATLAB.

Fluorescence Intensity Traces. Fluorescence intensity plots were obtained using the Twin Slicer application of Huygens Essential deconvolution software. One z-slice within the middle third of each nucleus was selected for analysis, and fluorescence intensity was traced along a single diameter within this z-slice. The location relative to the nuclear periphery was determined by selecting a z-slice in the middle third of the nucleus in which the allele focus was brightest. Lines then were traced through the brightest portion of the focus to the nearest point on the nuclear lamina.

Correlation Analysis. Correlation analysis was conducted on raw image stacks. Pearson’s correlation coefficient, r, was calculated as a measure of the average colocalization between two markers within cells. To restrict the analysis to cells which had been properly labeled, as well as to get an idea of inter-cell variability, we calculated r for individual cells that were selected based on nuclear structure and the presence of each marker. To this end, individual nuclei in the field of view were segmented from 3D image stacks using laminB1 to mark nuclear boundaries. All nuclear boundaries in the field of view were identified using a fixed threshold of laminB1 signal. The resulting binary image was segmented into individual nuclei using a connected components identification algorithm (67) combined with morphological closing (68). Correlation was calculated in the interior of each identified nucleus. Image processing was carried out in MATLAB.

To compare colocalization in PolII vs. H3K4me3 against the colocalization in PolII vs.RAG2, we fit a model of the form:

$$r_{ij} = m^+ + m^-H_i + f_i + e_{ij}$$

where $r_{ij}$ is the Pearson’s correlation (whole-cell colocalization) for the j-th field in the i-th field of view (i = 1, . . . , 10). The baseline mean (for PolII vs. H3K4me3) is denoted by $m^+$. For fields of view i in the PolII vs.RAG2 experiments, the term $m^-H_i$ denotes the difference of the mean colocalization between PolII vs.RAG2 compared with the colocalization between mPolII vs. H3K4me3. The term $f_i$ is an effect which captures the variability caused by the i-th field of view. It is assumed to be random and to have a Gaussian distribution with 0 mean and constant $\sigma_f$. Finally, $e_{ij}$ denotes measurement error. The model is fitted using restricted maximum likelihood using the nlme package in the R computing platform.

ACKNOWLEDGMENTS. We thank S. Johnson and Y. Gao of the Duke Comprehensive Cancer Center Light Microscopy Facility for imaging support, F. W. Alt for providing DiI/DijI mice, and B. Jones-Mason and H.-Y. Shih for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants R01 AI49934 (to M.S.K.), R37 AI32524 (to D.G.S.), and R01 CA12595 (to C.H.B.).


