The RAG2 C-terminus and ATM protect genome integrity by controlling antigen receptor gene cleavage

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Tight control of antigen-receptor gene rearrangement is required to preserve genome integrity and prevent the occurrence of leukaemia and lymphoma. Nonetheless, mistakes can happen, leading to the generation of aberrant rearrangements, such as Tcra/d–Igh inter-locus translocations that are a hallmark of ataxia telangiectasia-mutated (ATM) deficiency. Current evidence indicates that these translocations arise from the persistence of unreppaired breaks converging at different stages of thymocyte differentiation. Here we show that a defect in feedback control of RAG2 activity gives rise to bi-locus breaks and damage on Tcra/d and Igh in the same T cell at the same developmental stage, which provides a direct mechanism for generating these inter-locus rearrangements. Both the RAG2 C-terminus and ATM prevent bi-locus RAG-mediated cleavage through modulation of three-dimensional conformation (higher-order loops) and nuclear organization of the two loci. This limits the number of potential substrates for translocation and provides an important mechanism for protecting genome stability.
and T lymphocyte development is driven by V(D)J recombination, a process through which V, D and J gene-coding segments within each of the seven antigen receptor loci are rearranged to create a vast repertoire of antigen receptors\(^1,2\). Generation of receptor diversity through recombination is critical for shaping the adaptive arm of the immune system, enabling B and T cells to mount a focused and specific response to foreign antigen. This programmed rearrangement event relies on the lymphoid-specific proteins, RAG1 and RAG2 (recombination activating genes 1 and 2), which individually harbour many distinct regulatory domains whose functions remain largely enigmatic. Nonetheless, it is known that at least some of these contribute to the performance of RAG through fine-tuning of targeting, cleavage and repair. Furthermore, the proper functioning of the recombinase complex relies on cooperation between the two proteins, RAG1 and 2.

Specificity of targeting is conferred by RAG1-mediated recognition of highly conserved recombination signal sequence (RSS) elements that flank the individual V, D and J gene-coding segments, which are arrayed along each antigen receptor locus\(^3,4\). Moreover, RAG1 carries the catalytic endonuclease activity\(^4,5\).

However, cleavage cannot occur in the absence of its partner protein, RAG2 (refs 6,7), which contains a plant homeo domain that is known to directly bind the recombinase to active chromatin through recognition of the histone modification, H3K4me3 (refs 8,9). The RAG1/2 complex binds to two gene segments (that can be many kilobases apart), brings them together and cuts at the RSS borders to generate DNA double-strand breaks (DSBs). Following cleavage, the four resulting broken ends are held together in a RAG post-cleavage complex that is instrumental in directing repair by the ubiquitous non-homologous end joining pathway\(^10,11\). The introduction of DSBs activates several PI3K-like Ser/Thr kinases, including the ataxia telangiectasia-mutated (ATM) kinase, which phosphorylate downstream downstream proteins and orchestrate the DNA-damage response\(^2\). Other DNA-damage response factors, like the histone variant γ-H2AX, 53BP1 (p53-binding protein 1) and the MRN complex (containing Mre11, Rad50 and Nbs1), are rapidly recruited and form nuclear foci at the site of DSBs\(^2,11\).

Recombination is tightly regulated so that the appropriate loci and gene segments are rearranged in the appropriate lineage (T-cell receptor (Tcr) loci in T cells and immunoglobulin (Ig) loci in B cells) and at the appropriate developmental stage. In T cells, productive rearrangement of the different Tcr loci gives rise to two distinct lineages: recombination of Tcrγ/Tcrδ and Tcrβ/Tcrα leads to γδ- and γβ-T cells, respectively\(^12,13\). Despite this separation, recombination of the different loci overlaps. Tcrγ, Tcrδ and Tcrβ are all rearranged at the early CD4-CD8- double-negative (DN) 2/3 stage of development, whereas Tcrα recombination occurs later in double-positive (DP) cells\(^14\).

Regulation of Tcrδ and Tcrα recombination is uniquely complicated because, beyond the fact that they recombine at different stages of differentiation, Tcrα and Tcrδ share the same chromosomal location, with the latter embedded between the Vα and Jα gene segments. Furthermore, promiscuous Dα→Jα rearrangement of the Igh locus, which occurs at low level in T-lineage cells\(^15\), adds yet another layer of complexity. Together, these issues compound the risks associated with Tcrα/d recombination and the probability of aberrant repair. Indeed, inter-locus rearrangements between Tcrα/d and Igh have been identified as a hallmark of thymic lymphomas in ATM-deficient mice\(^16\). Moreover, we recently discovered translocations between these two loci associated with an absence of the non-core C-terminal domain of RAG2 (ref. 17). Although this domain is dispensable for recombination\(^18,19\), its deletion is known to affect the joining step, as well as the order, efficiency and fidelity of the reaction\(^20–25\). When coupled with the disruption of p53, we found that Rag2\(^{−/−}\) p53\(^{−/−}\) mice develop thymic lymphomas harbouring recurrent translocations involving Tcrα/d and Igh, defects that are similar to those found in Atm\(^{−/−}\) mice\(^12\). In addition, ATM and the C-terminus of RAG2 have similar defects in stabilizing the RAG post-cleavage complex\(^17,26\).

Here we have now investigated the mechanisms underlying the origins of the inter-locus Igh–Tcrα/d translocations before lymphomagenesis to determine whether regulation of cleavage and nuclear accessibility of the loci is perturbed by an absence of ATM and the C-terminus of RAG2. We find that Igh cleavage occurs at higher levels in DN2/3 versus DP cells, and thus its rearrangement could overlap with Tcrα/d rearrangement. However, breaks are not found in Igh and Tcrα/d in the same cell, except in the absence of the RAG2 C-terminus or ATM. Control of mono-locus cleavage involves regulated mono-locus looping out from the chromosome territory and mono-locus association with repressive pericentromeric heterochromatin (PCH). In the absence of the RAG2 C-terminus or ATM, nuclear accessibility is increased and both loci remain euchromatin, and bi-locus cleaves can be detected coincident with bi-locus cleavage. Interestingly, we found that expression of RAG brings Tcrα/d and Igh into close proximity in DN2/3 cells (when RAG-mediated cleavage of Igh occurs at high levels), whereas the two loci separate at the subsequent DP cell stage of development (when recombination of Igh is reduced). In contrast, in the mutant cells, increased association of Tcrα/d-Igh in DP cells is linked to increased nuclear accessibility, and the introduction of bi-locus breaks and damage on proximal loci. In sum, this study show a role for the non-core domain of RAG2 and ATM in controlling recombination between two loci via modulation of nuclear organization. Moreover, these events provide a direct mechanism for the generation of the Tcrα/d-Igh translocations that are found in Rag2\(^{−/−}\) p53\(^{−/−}\) and Atm\(^{−/−}\) tumours.

**Results**

**Igh and Tcrα/d rearrangement overlaps in DN2/3 and DP cells.** Although it is established that the Igh locus (which is located on chromosome 12) undergoes low-level D\(\text{H}_1\)–\(\text{H}_1\) rearrangement (at the 3′ end of the locus) in T cells, it is not known at which stage of development recombination occurs. To determine this, we performed an immuno-DNA fluorescence in situ hybridization (FISH) experiment to analyse RAG-mediated cleavage on Igh in wild-type DN2/3 and DP cells (Fig. 1a) using a DNA probe that hybridizes to the 3′ end of this locus (see Fig. 1b) in combination with an antibody against the phosphorylated form of H2AX, γ-H2AX, as a read-out for double-stranded breaks (DSBs)\(^27,28\).

Our analyses showed that RAG-mediated γ-H2AX foci were associated more frequently with Igh in DN2/3 compared with DP cells, suggesting that D\(\text{H}_1\)–\(\text{H}_1\) rearrangement occurs predominantly, but not exclusively, at the earlier stage of development (Fig. 1c and Supplementary Table S1). In contrast, RAG-mediated γ-H2AX foci were associated more frequently with Tcrα/d in DP compared with DN2/3 cells (Fig. 1c and Supplementary Table S1).

The RAG2 C-terminus and ATM regulate mono-locus cleavage. As both Tcrα/d and Igh loci undergo recombination in T cells, it is conceivable that there is an overlap in the timing of their rearrangement. If so, bi-locus breaks could be introduced concurrently in the same cell, and, in the event of a defect in joining, these could act as substrates for translocations. However, when we examined the frequency of γ-H2AX foci associated with both Tcrα/d and Igh (bi-locus breaks) in individual wild-type DN2/3 and DP cells, we found these present at a very low level, which was only slightly above the frequency of bi-locus breaks.
detected in RAG-deficient cells (Fig. 2a and Supplementary Tables S2 and S3). In contrast, we found a significant rise in the frequency of bi-locus breaks in individual Rag2\textsuperscript{c/c} p53\textsuperscript{−/−} or Atm\textsuperscript{−/−} cells, with the most pronounced increase occurring at the DP stage, at the time of Tcra recombination (Fig. 2a, b and Supplementary Tables S2 and S3). It is of note that we found no increase in the frequency of bi-locus breaks in the absence of another DNA-damage response factor, 53BP1, which is known to be important for the long-range joining of coding ends in V(D)J recombination\textsuperscript{29} (Fig. 2a and Supplementary Tables S2 and S3). These data indicate that bi-locus breaks in the same cell do not occur simply as a result of a defect in repair. Because cells deficient in both p53 and ATM have defects in cell cycle checkpoints that enable unrepaired breaks to be propagated as a result of cell division\textsuperscript{30,31}, it is possible that some proportion of the bi-locus breaks that we detected in mutant DP cells could be attributed to the persistence of unrepaired breaks being amplified in proliferating DN4 cells.

Figure 1 | Igh and Tcra/d rearrangement overlaps in DN2/3 and DP T cells. (a) Scheme of T-cell development with stages of recombination of the four Tcr loci. (b) Scheme representing the Tcra/d and Igh loci with probes for 3D DNA FISH shown below. (c) Frequency of γ-H2AX association on Igh or Tcra/d alleles in wild-type (WT) DN2/3 and DP cells. P-values were calculated using a two-tail Fisher’s exact test, *significant (5.00e-2>P≥1.00e-2) and ***highly significant (P<1.00e-3)). Experiments were repeated at least twice, and data are displayed as a combination of two independent experimental sets (n>500 for each stage/genotype; see Supplementary Table S1 for details and individual data sets).

Figure 2 | The RAG2 C-terminus and ATM regulate mono-locus cleavage. (a) Frequency of γ-H2AX association on both Tcra/d and Igh loci in individual wild-type (WT), Rag1\textsuperscript{−/−} (R1\textsuperscript{−/−} or R1\textsuperscript{−/−} β (see Methods section for details)), S3bp\textsuperscript{−/−}, Atm\textsuperscript{−/−}, RAG2\textsuperscript{c/c} and RAG2\textsuperscript{c/c} p53\textsuperscript{−/−} DN2/3 and DP cells. (b) Confocal sections showing a representative example of γ-H2AX association (in yellow) on both Tcra/d and Igh loci (3′α in red, 3′Igh in blue). Scale bar, 1μm. P-values were calculated using a two-tail Fisher’s exact test (ns, no significance (P≥5.00e-2), *significant (5.00e-2>P≥1.00e-2), **very significant (1.00e-2>P≥1.00e-3) and ***highly significant (P<1.00e-3)). Experiments were repeated at least twice, and data are displayed as a combination of two independent experimental sets (n>200 for each stage/genotype; see Supplementary Tables S2 and S3 for details and individual data sets).
which mark the transition from the DN2/3 to the DP stage of development (Fig. 1a). To check this, we compared the frequency of bi-locus breaks in individual \( \text{Rag}^{2/c} \) versus \( \text{Rag}^{2/c} \ p53^{-/-} \) cells. However, we found no significant differences in the incidence of bi-locus breaks in the two genotypes, and so we conclude that the increase in bi-locus breaks that we detected in \( \text{Rag}^{2/c} \ p53^{-/-} \) DP cells does not result from an absence of p53 (Fig. 2a and Supplementary Tables S2 and S3). Indeed, we also found no significant increase in bi-locus breaks on \( \text{Tcra}^{d} \) and \( \text{Igh} \) in wild-type versus \( p53^{-/-} \) cells (4% versus 4.6%, respectively). In addition, differences in the level of ATM in \( \text{Rag}^{2/c} \) versus wild-type cells cannot explain the differences in the frequency of bi-locus breaks, as western blot analysis showed similar levels of protein in the two genotypes (Supplementary Fig. S1). Taken together, these data indicate that the C-terminus of RAG2 and ATM regulate cleavage to ensure that RAG-mediated breaks are introduced on only one locus at a time in each recombining T cell.

The RAG2 C-terminus and ATM regulate \( \text{Tcra-Igh} \) association. We and others have shown that nuclear proximity of broken partner genes is an important factor in translocations$^{32–37}$. As our studies indicate that translocations between \( \text{Tcra}^{d} \) and \( \text{Igh} \) are found in tumours from \( \text{Rag}^{2/c} \ p53^{-/-} \) and \( \text{Atm}^{-/-} \) mice$^{17}$, we wanted to examine the association between the two loci in recombining DN2/3 and DP cells. For this, we performed circularized chromosome conformation capture with next-generation sequencing (4C-seq), using a bait sequence spanning the \( \text{Tcra} \) enhancer (E) at the 3' end of the locus (see Methods section for details; Fig. 3a, Supplementary Fig. S2a and S2b) to generate a heat map of the \( \text{Tcra-Igh} \) association across the entire chromosome 12mino. To achieve this, we performed ChIP-seq to determine the location of relevant marks in DN2/3 and DP cell lines. To ensure consistency, we also used a window size of 30 Mb to capture both inter-locus and intra-locus distances.

Figure 3 | The RAG2 C-terminus and ATM regulate \( \text{Tcra-Igh} \) association. (a) Domainograms showing a heatmap of \( \text{Tcra}^{d} \) interactions across the entire chromosome 12 (left) and the \( \text{Igh} \) locus (right) using a window size of 30 HindIII sites in DN2/3 (top) and DP (bottom) cells. See also Supplementary Fig. S2 and Supplementary Table S4. (b) Confocal sections showing representative examples of unpaired (top) and paired (bottom) loci: 3' in red, 3'Igh in blue. Scale bars, 1μm. (c) Alignment of ChIP-seq data at the \( \text{Igh} \) locus showing levels of enrichment of H3K4me3 (green), H3K9ac (red) and RAG2 binding (purple)$^{39}$ in DN and DP cells. See also Supplementary Table S5. (d,e) Cumulative frequency curves of \( \text{Tcra-Igh} \) inter-locus distances in: wild-type (WT) and \( \text{R1}^{-/-} \) DN2/3 cells (d), WT, \( \text{S3bp1}^{-/-} \), \( \text{Atm}^{-/-} \) and \( \text{Rag2}^{2/c} \ p53^{-/-} \) DN2/3 (e, left) and DP (e, right) cells (cut-off at 1.5 μm). A left shift indicates closer association. P-values were calculated using a two-tail Fisher’s exact test (ns, no significance (\( P \geq 0.006 \)) **very significant (1.00e-2 > \( P \geq 1.00e-3 \)) and ***highly significant (\( P < 1.00e-3 \))_. Experiments were repeated at least two times, and data are displayed as a combination of two independent experimental sets (\( n > 200 \) for each stage/genotype).
Supplementary Table S4). The 4C data are displayed as a domainogram, which is a statistical way to visualize local 4C signal enrichment across a region of interest39. It is clear from Fig. 3a (left panels) that there are significant changes in the interaction partners of Tcra/d in the two populations across chromosome 12. Importantly, the intensity of the Tcra/d-Igh interaction is much higher in DN2/3 cells where, according to our γ-H2AX analysis, the latter is recombined at higher frequency (Fig. 3a (right panels)). This result was validated by additional three-dimensional (3D) DNA FISH experiments, and distances separating the two loci were plotted as cumulative frequency curves, using a cut-off of 1 μm to measure close association (that is, ‘pairing’) (Supplementary Fig. S2b). On these graphs, a left shift is indicative of closer association. Examples of paired and unpaired loci are shown in Fig. 3b.

Previous studies indicate that RAG binds to active chromatin and localizes to the J segments at the 3′ end of each antigen receptor locus in rearranging cells39. We have recently shown that localized RAG enrichment in this region is linked with homologous Tcra pairing, transcription and regulated mono-allelic cleavage in DP cells40. Thus, we wanted to investigate whether the presence of RAG could influence the frequency with which Tcra/d and Igh contact each other during recombination. There are two regions of RAG2 binding on the Igh locus in developing T cells (Fig. 3c). The first corresponds to enrichment of H3K4me3 and H3K9ac at the 3′ end of Igh, where D11–J11 rearrangement is known to occur in these cells15. In addition, RAG2 also binds to the V14 portion of Igh, and this could explain the high frequency of interaction with Tcra in this region (Fig. 3a,c and Supplementary Table S5). Interestingly, we found that an absence of RAG1 significantly decreased heterologous association of the two loci in DN2/3 cells, whereas we observed no significant change in DP cells (Fig. 3d and Supplementary Fig. S2c). These data indicate that RAG1 brings recombining Tcra/d and Igh together in DN2/3 cells (where Igh cleavage occurs at high levels) but that these loci separate in DP cells (where cleavage of Igh is reduced and cleavage of Tcra occurs at high levels) (Fig. 1c). Intriguingly, the absence of the C-terminus of RAG2 or ATM deficiency did not affect the frequency of Tcra/d-Igh association in DN2/3 cells; however, in DP cells, where association of the two loci is normally reduced, we observed an increase in the incidence of heterologous pairing in both mutants, although pairing in the equivalent 53BP1-deficient populations followed the same pattern as in wild-type cells (Fig. 3e). Importantly, bi-locus breaks were increased on heterologously paired loci in Rag2+/+ /C0 p53−/− and Atm+/− /C0 p53−/− DP cells compared with wild-type control and 53BP1-deficient cells (Fig. 4a,b and Supplementary Table S6). These data indicate that an increased frequency in contact between Tcra and Igh in Rag2+/+ /C0 p53−/− and Atm+/− /C0 p53−/− DP cells is linked to an increased frequency in bi-locus breaks on paired loci.

**RAG-mediated pairing is not a general phenomenon.** Genomewide analysis has shown that RAG2 has an overlapping pattern with H3K4me3-enriched regions39; thus, we wanted to determine whether other RAG-enriched active genes would pair up with Tcra in the same way as Igh. For this analysis, we selected five hematopoietic lineage-specific genes (Hmbg1, Ldt, Catg, Ly6d and Sabt1), as well as a housekeeping gene (Gapdh), which are all located on different chromosomes and transcribed in these cells (Fig. 5a,b). Interestingly, pairing of Tcra with these other RAG-enriched loci shows an opposite trend to Igh–Tcra pairing in all cases (Fig. 5c and Supplementary Table S7). Indeed, in contrast to Tcra–Igh, an absence of RAG1 appears to increase their contact frequency with Tcra in DN2/3 cells, and association is even more pronounced at the DP stage of development. Furthermore, pairing between these control loci follows the same trend as their association with Tcra (Fig. 5d and Supplementary Table S7). It is of note that the maximum frequency of pairing (for individual pairs of alleles) in all cases is very similar (around 20%), and this matches the maximum level of homologous Tcra pairing seen during recombination in DP cells40. Taken together, these data indicate that the trend for Tcra–Igh is unique, and even though RAG2 is enriched on control loci, pairing does not depend on the presence of RAG. Instead, interactions between control loci may be influenced by binding of other transcription factors that are involved in their regulation. In this context, the Fraser lab has indicated that transcription-factor-bound regions determine intra- and inter-domain interactions that are developmentally regulated41.42. Thus, perhaps other transcription factors that are enriched on these control genes may be dominant in determining their interaction partners in developing T-cell nuclei.

**Regulation of RAG cleavage is linked to genome stability.** The Tcra and Igh loci are located on different chromosomes, namely
DNA FISH for in bi-locus loop formation on paired most breaks (Fig. 4a). In contrast, there was a significant increase and Supplementary Table S8), the locus in which we detected the locus at a time, and these predominantly involved vast majority of higher-order loop formation occurred on one were paired. Our analyses indicate that in wild-type DP cells the association between these two loci occurs in nuclear space, we focused our attention on the formation of higher-order loops that move genes outside of their chromosome territories as a mechanism by which they could be brought into close contact. We recently showed that mono-locus looping out of Tcra and Igh from their respective chromosome 14 and 12 territories when the loci were paired. Our analyses indicate that in wild-type DP cells the vast majority of higher-order loop formation occurred on one locus at a time, and these predominantly involved Tcra (Fig. 6a,b and Supplementary Table S8), the locus in which we detected the most breaks (Fig. 4a). In contrast, there was a significant increase in bi-locus loop formation on paired Tcra–Igh alleles in Rag2−/− p53−/− and Atm−/− DP cells (Fig. 6a,b and Supplementary Table S8), coincident with an increase in bi-locus breaks (Fig. 4a). Taken together, our data indicate that an increase in Tcra–Igh pairing in Rag2−/− p53−/− and Atm−/− DP cells is linked to an increase in bi-locus looping and bi-locus breaks on paired alleles.

Higher-order looping of genes away from their chromosome territories has previously been correlated with open chromatin and an active transcriptional status, while silent genes are positioned more internally43–46. To determine whether nuclear accessibility of the individual loci is linked to loop formation, we examined the location of paired Tcra–Igh relative to the repressive pericentric heterochromatin (PCH). In wild-type DP cells, we found that paired Tcra–Igh alleles were frequently located at PCH; however, the two loci were not equivalently close (Fig. 6c,d and Supplementary Table S9). Interestingly, we found that the Igh locus was predominantly in contact with PCH, whereas Tcra, the locus associated with the most loops and breaks

Figure 5 | RAG-mediated pairing is not a general phenomenon. (a) Alignment of ChIP-seq data at the Hmgb1, Gapdh, Lat, Cd3g, Ly6d and Satb1 loci showing levels of enrichment of H3K4me3 (green), H3K9ac (red) and RAG2 binding (purple)39 in DN and DP cells. (b) Confocal sections showing 3D DNA FISH for Tcra and three control loci. 3′s in red, Hmgb1 in yellow, Lat in purple and Ly6d in green. Scale bar, 1μm. (c,d) Graphs showing the frequency of heterologous pairing (inter-locus distance <1μm) between Tcra and the control loci (c), or between the loci (d). Experiments were performed at least one time, and data are displayed as a combination of independent experimental sets when applicable (n>200 for each stage/genotype; see Supplementary Table S7 for details).
in these pairs, remained euchromatic. In contrast, in \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) DP cells, repositioning of the \( \text{Igh} \) to PCH was significantly reduced, and this increased nuclear accessibility was associated with increased bi-locus breaks (Fig. 6c,d). These data indicate that the increase in bi-locus breaks on paired \( \text{Tcra}–\text{Igh} \) alleles in \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) DP cells is linked with an increase in the simultaneous nuclear accessibility of the two loci, as assessed by looping out from their chromosome territories and by their location away from repressive PCH.

Because close proximity of broken partner loci in DP cells could provide a fertile ground for the generation of the aberrant inter-locus rearrangements that are a characteristic of \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) lymphomas, we next asked whether the increase in bi-locus breaks and increased accessibility of paired \( \text{Tcra}–\text{Igh} \) was linked with an increase in bi-locus instability in interphase \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) cells before the onset of lymphomagenesis. For this, we performed a DNA FISH experiment using DNA probes that hybridize outside the 3′ and 5′ ends of each locus (see Fig. 1b) and examined the frequency of damage (that is, split alleles (>1.5 \( \mu \)m in between the two ends), duplicated or missing signals). The increase in bi-locus damage in individual \( \text{Tcra}–\text{Igh} \) was very significant (Fig. 6e,f and Supplementary Table S10). Thus, an absence of the C-terminal domain of \( \text{Rag2} \) or ATM results in an increase in both bi-locus breaks and bi-locus damage in the same cell.

**Discussion**

Taken together, our data show that translocations between \( \text{Tcra} / \text{Igh} \) that are found in tumours from \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) mice may arise not only as a result of \( \text{Tcra} \) recombination (as shown previously in ATM-deficient mice \(^{16}\)) but also from \( \text{Tcra} \) recombination. It has previously been proposed that the aberrant inter-lobe \( \text{Tcra}–\text{Igh} \) rearrangements found in \( \text{Atm}^{-/-} \) T cells arise from the persistence of unrepaird breaks converging at different stages of thymocyte differentiation.\(^{16}\) However, in contrast to our analyses, these studies did not examine breaks on \( \text{Tcra} / \text{Igh} \) in individual developing T cells, but rather they analysed translocations in \( \text{Atm}^{-/-} \) mature T cells and \( \text{Atm}^{-/-} \)-derived thymic lymphomas, respectively. Here we show that breaks on the two loci occur in the same cell at the same stage of development, which provides a direct mechanism for the generation of the characteristic inter-locus \( \text{Tcra}–\text{Igh} \) translocations that are found in \( \text{Rag}^{2/c} \) \( p53^{-/-} \) and \( \text{Atm}^{-/-} \) T lymphomas.

Our studies reveal that there is similarity between the C-terminal of \( \text{Rag2} \) and ATM in temporally harnessing RAG activity to ensure that cleavage occurs on only one locus at a time in recombining T cells. Intriguingly, we find that RAG-mediated association of recombining loci in localized ‘recombination centres’ is linked to feedback control of RAG cleavage: the introduction of a break on one locus is coupled with repositioning of the partner locus to PCH and inhibition of bi-locus cleavage and looping out from the territory (see Model in Supplementary Fig. S3). Targeting of RAG to the correct locus (\( \text{Tcra} \) rather than \( \text{Igh} \)) is likely to be influenced by the higher level of transcription on \( \text{Tcra} \). Interestingly, although cleavage of \( \text{Tcra} \) is reduced in paired \( \text{Tcra}–\text{Igh} \) alleles in \( \text{Rag}^{2/c} \) \( p53^{-/-} \) DP cells (perhaps due to a decrease in efficiency of cleavage in the absence of the
C-terminus of RAG2), there is a significant increase in cleavage of Igh, which further underlines the role of regulation in trans between the two loci. Although we recently showed that pairing and higher-order looping of Tcra homologous alleles are linked with regulation of their recombination[40], cross-talk and regulation of heterologous loci in trans have not previously been shown to be linked to the formation of higher-order looping. Indeed, higher-order loop formation was previously shown to be involved in stochastic interactions between different loci on separate chromosomes[41,49], although we have found here that interactions between Tcra/d and Igh are mediated by the presence of RAG.

Together, our data suggest that regulation of mono-locus cleavage reaps changes in nuclear organization that are associated with a reduction in accessibility. Regulation of mono-locus cleavage is thus akin to regulation of mono-allelic cleavage. Indeed, our previous studies showed that ATM recruited to the site of a break on one allele acts in trans to reposition the second homologous allele to PCH and to prevent the introduction of further breaks on the partner homologue. We propose that homologous and heterologous antigen receptors come together in localized recombination centres for coordinated regulation of RAG cleavage. Here we have now identified an auto-regulatory role for RAG2 in restricting cleavage in an analogous manner to ATM. These data explain the mechanisms underlying the origins of the inter-locus Igh–Tcra/d in Atm−/− and Rag2−/− p53−/− lymphocytes before lymphomagenesis. Importantly, they identify an unappreciated role for the C-terminal domain of RAG2 in regulating chromosome dynamics and accessibility of target loci to restrict ongoing cleavage after the introduction of a break on one locus. Thus, feedback control of RAG activity relies on signals transmitted via the RAG complex itself.

Regulation of V(D)J recombination occurs at multiple levels to prevent the occurrence of chromosomal translocations or deletions that can result from errors in repair and/or mis-targeting of RAG1/2 to cryptic RSSs. However, beyond degradation of RAG2 protein[31] and our more recent findings that expression of Rag1 is regulated across cell cycle[32], there have been no studies that focus specifically on autoregulation of RAG cleavage activity in individual cells. It is clearly critical to have such mechanisms in place to ensure that further breaks are not introduced in cis or trans on accessible target loci that undergo recombination at overlapping stages of development (Tcrg, Tcrb, Tcra/d and Igh in DN T cells and Igh and Igk in pre-B/immature B cells). Furthermore, this same mechanism could also be important in preventing cleavage on actively transcribed off-target loci with cryptic RSSs that bind RAG.

Methods
Mice. The Atm−/−, Rag2−/− and Rag2−/− p53−/− mice were provided by Deriano et al.[37] RAG1-deficient mice were provided by Yanhong Li, Grace Teng and David Schatz. Rag1−/− deficient cells (Pr1−/− – β and control WT) were derived from mice carrying a functionally rearranged Tcrg transgene (β) that allows T-cell development to proceed to the DP stage in the absence of Tcrg rearrangement. The SBP1−/− mice were provided by Davide Robbiani and Michel Nussenzweig[33]. Wild-type littermates were used as controls. Animal care was approved by the NYU School of Medicine Animal Care and Use Committee of (protocol number 120315-01).

T-cell flow-cytometry sorting. Flow-cytometry cell sorting was performed on a MoFlo or Reflection sorter. Antibodies were as follows: Thy1.2 PE-Cy7 (CD90.2, clone 53-2.1, 1:000 dilution; eBioscience), TCRγ/δ APC-eFlour780 (clone H57-597, 1:500 dilution; eBioscience), CD4 APC (L3T4, RM4-5, 1:500 dilution, BD Biosciences), CD8a FITC (clone 53-6.7, 1:500 dilution, BD Biosciences) and CD25 PE (PC61, 1:500 dilution, BD Biosciences). The gating strategy was: Thy1.2− / TCRγδ+ / CD4− / CD8− for DP cells and Thy1.2− / TCRγδ− / CD4+ / CD8+ for DN2/3 cells.

3D DNA FISH and immuno-FISH. 3D DNA FISH and combined DNA FISH-immunofluorescence for γ-H2AX (immuno-FISH) were carried out on T cells adhered to poly-l-lysine-coated coverslips, as previously described[54,55]. Briefly, cells were fixed with 2% paraformaldehyde/PBS (pH 7.7–4) for 10 min at room temperature (RT) and permeabilized for 5 min with 0.4% Triton/PBS on ice. After 30 min of blocking in 2.5% BSA/10% normal goat serum/0.1% Tween-20/PBS, cells were sequentially incubated with a primary antibody against phosphorylated histone H2AX (γ-H2AX, MAB2872, Cell Signaling) and a secondary goat-anti-mouse antibody (Alexa Fluor 488 or 555; Invitrogen) for 1 h each at RT. Cells were then post-fixed in 2% paraformaldehyde/PBS for 10 min at RT, incubated with 0.1 mg ml−1 RNaseA for 30 min at 37°C and permeabilized in 0.7% Triton X-100/0.3 M HCl for 10 min on ice. Cells were then denatured with 50% formamide/2 × SSC (pH 7.7–4) for 30 min at 80°C and hybridized overnight with the probes at 37°C. The next day, cells were rinsed three times in 50% formamide/2 × SSC and three times in 2 × SSC at 80°C for 5 min each. Finally, slides were mounted in ProLong Gold (Invitrogen) containing 1.5 μg ml−1 4',6-diamidino-2-phenylindole (DAPI).

Probes. BAC probes RP23-25SN11 (3′ Tcra), RP23-304L2 (5′ Tcra), CT7-3416 (3′ Igh), RP23-3461617 (5′ Igh), RP23-289Q10 (Hmgil), RP23-358H24 (Lpr5), RP23-277H19 (Lpr6) and RP23-137H17 (Satb1) were directly labelled by nick translation with ChromaTide Alexa Fluor 488 or 594-5-UTP (Molecular Probes) or Cy3- or Cy5-dUTP (Fisher). For one copy, 0.5 μg of nick-translation product was precipitated and resuspended in 10 μl of hybridization buffer (50% formamide/20% dextran sulphate/× Denhardt’s reagent, 0.2 mg/ml hoescht 33258, 95°C pre-annealed for 45 min at 37°C before overnight hybridization with cells. XCyting Mouse Chromosome 14 (Texas-red) and 12 (FTTC) paints (Metaphase Systems) were separately followed separately following the supplier’s instructions. Paint and BAC probes were combined just before overnight hybridization.

Confocal microscopy and analysis. 3D images were acquired by confocal microscopy on a Leica SP5 Acheson-Optical Beam splitter system. Optical sections separated by 0.3 μm were collected, and stacks were analysed using Image J software. Alleles were defined as associated with γ-H2AX if the BAC signals and immunofluorescence foci were at least partially overlapped (at least one pixel of colocalization). Alleles were considered as located at PCH when BAC signals were adjacent or overlapping with a γ-satellite signal (no pixel in between the edges of the BAC and γ-satellite signals). Distances from the loci to their chromosome territories (higher-order looping) were measured from the centre of mass of the BAC signal to the closest edge of the chromosome paint. Distances between alleles or loci were measured between the centre of mass of each BAC signal.

Statistical analyses. The statistical tests were applied to combined data sets from related experiments. Supplementary Tables display individual experiments to show the low level of variation between the repeats. Statistical analyses were performed using a two-tail Fisher’s exact test: P-values ≤ 5.00e-2 (a = 0.05) were taken to be significant (1.00e−2 ≤ P ≤ 5.00e−2, *significant; 1.00e−3 ≤ P ≤ 1.00e−2, **very significant; and P ≤ 1.00e-3, ***highly significant).
DpnII digestion: HindIII-ligated 3C template was digested overnight at 37 °C with DpnII (50 μl 10 × restriction buffer, 50 U DpnII and 300 μl sterile water). DpnII was inactivated at 65 °C for 30 min.

Ligation and precipitation: DNA was ligated overnight at 16 °C in 12 ml sterile water, 1.4 ml ligation buffer and 100 U ligase. Ligation products were phenol-extracted and ethanol-precipitated using glycogen as a carrier (20 mg ml⁻¹), and they were resuspended in 15 ml 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (pH 8.0), 0.3% SDS. Samples were treated using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

PCR reaction and sequencing: Specific primers for the Tera Ex enhancer were HindIII 5'-AGACAGACCTCGGGAAATGTT-3' and DpnII 5'-TAAAGTCGACGACAGATG-3'. The Illumina-specific adapter sequences were included at the 5'- end of each primer. PCR reactions were performed using the Expand Long Template PCR system (Roche), and PCR conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 68 °C for 3 min; followed by a final step of 68 °C for 7 min. The 4C library was sequenced on an Illumina GAIIx single-read 72-cycle run. 4CChIP analysis: The 45-bp single-end reads were aligned to a library of 40-bp flanking regions of HindIII restriction sites, on the mouse genome sequence (build mm9). The alignment was performed using Maq software (http://maq.sourceforge.net/) with a quality threshold of 150. We kept the distinction between the upstream and downstream flanking region of each HindIII site. We segmented the genome according to the HindIII restriction sites and removed those HindIII sites consisting of two consecutive HindIII restriction sites that did not contain a DpnII restriction site.

Domainogram analysis: We followed the analytical steps described previously38, using the genomic regions enclosed between two contiguous HindIII sites. We then used windows of increasing size extending up to 30 HindIII sites. We computed and analysed scores of each window size as follows: first, we scored each pair of contiguous HindIII sites (defined as Q(x) = (rank(x) – 0.5)/N, where Q is a quartile score of HindIII sites (x) and N is the total number of these sites). The Q-values are calculated in a global manner for the whole genome, with the bait chromosome analysis performed separately. We computed the combination of scores within each window to a form that was amenable to applying Fisher’s approach for combining independent tests of significance. The range of colours in the domainograms represents the intensity of these scores. See Supplementary Table S4 for details.

Chromatin immunoprecipitation sequencing: Chromatin immunoprecipitation sequencing (ChIP-seq) preparation was carried out as previously described26, and ChIP-seq analysis was performed using the Qseq algorithm27 (details were provided in Chaumel et al.46).

Cell fixation and lysis: A total of 1 × 10⁶ cells were fixed in 1% formaldehyde for 10 min at RT and lysed in 15 mM Tris pH 7.5, 60 mM KCl, 15 mM NaCl, 15 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose and 0.3% NP-40 for 10 min at 4 °C. Nuclei were isolated by centrifugation and washed once in ‘digest buffer’ (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.5% SDS) and resuspended in 75 μl dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA (pH 8.0), 16.7 mM NaCl, 150 μM glycogen, 10 μM TE and 100 U ligase). Ligation products were phenol-extracted and ethanol-precipitated using glycogen as a carrier (20 mg ml⁻¹). Blots were developed with enhanced chemiluminescence (Pierce).

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