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Accessibility of Chromosomal Recombination Breaks in Nuclei of Wild-type and DNA-PKcs-Deficient cells

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Abstract

V(D)J recombination is a highly regulated process, proceeding from a site-specific cleavage to an imprecise end joining. After the DNA excision catalyzed by the recombinase encoded by Recombination Activating Genes 1 and 2 (*RAG1/2*), newly generated recombination ends are believed held by a post-cleavage complex (PC) consisting of *RAG1/2* proteins, and are subsequently resolved by non-homologous end joining (NHEJ) machinery. The relay of these ends from PC to NHEJ remains elusive. It has been speculated that NHEJ factors modify the *RAG1/2*-PC to gain access to the ends or act on free ends after the disassembly of the PC. Thus, recombination ends may either be retained in a complex throughout the recombination process or left as unprotected free ends after cleavage, a condition that may permit an alternative, non-classical NHEJ end-joining pathway. To directly test these scenarios on recombination-induced chromosomal breaks, we have developed a recombination end protection assay to monitor the accessibility of recombination ends to exonuclease-V in intact nuclei. We demonstrate that these ends are well protected in the nuclei of wild-type cells, suggesting a seamless cleavage-joining reaction. However, divergent end protection of coding *versus* signal ends was found in cells derived from severe combined immunodeficient (*scid*) mice that are defective in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). While signal ends are resistant, opened coding ends are susceptible to enzymatic modification. Our data suggests a role of DNA-PKcs in protecting chromosomal coding ends. Furthermore, using recombination-inducible *scid* cell lines, we demonstrate that conditional protection of coding ends is inversely correlated with the level of their resolution, i.e., the greater the accessibility of the coding ends, the higher level of coding joints formed. Taken together, our findings provide important insights into the resolution of recombination ends by error-prone alternative NHEJ pathways.

Keywords

DNA-PKcs; *RAG1*; *RAG2*; *scid*; V(D)J recombination

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Conflict of Interest.

None.

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1. Introduction

The variable regions of antigen-receptor genes, coding for immunoglobulins and T cell receptors, are assembled from variable (V), diversity (D) and joining (J) gene segments by a recombination reaction, known as V(D)J recombination. This recombination process occurs in two distinct, but coupled steps, a site-specific DNA excision followed by imperfect DNA end joining [1,2]. The site specific cleavage is dictated by the presence of conserved recombination signal sequences (RSS) flanking each V, D or J gene segment, which are targeted by lymphoid-specific recombinases encoded by the recombination activation genes, *RAG1* and *RAG2* [1]. *RAG1/2* proteins initiate recombination by recognizing a pair of RSS, catalyzing excision at the border between RSS and coding segments, generating hairpinned coding ends (CEs) and blunt signal ends (SEs) [3–5]. After cleavage, these ends are believed to remain associated with *RAG1/2* proteins, constituting the transitory structure referred to as the post-cleavage complex (PC) [6,7]. Subsequently, SEs and CEs are resolved into signal joints (SJs) and coding joints (CJs), respectively, by non-homologous end joining (NHEJ) proteins, including the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), Artemis, Ku70/80, XRCC4, Cernunnos/XLF and Ligase IV [8–12].

Although inactivation of any of these NHEJ genes results in defective V(D)J recombination and aborted lymphocyte development in mutant mice, the cells derived from these individual mutants are still capable of, albeit at very low levels, resolving some double stranded DNA breaks (DSBs), presumably by alternative NHEJ pathways [13]. Interestingly, this alternative pathway has also been observed in cells that express certain mutated forms of *RAG1* or *RAG2* [14,15]. These findings have led to a model in which functional *RAG1/2* direct recombination ends to the classical NHEJ pathway for efficient resolution. According to this model, either defective *RAG1/2* proteins that destabilize the PC or NHEJ mutants that fail to resolve the ends efficiently may expose recombination ends to alternative pathways or factors for aberrant resolution, including homologous recombination (HR) factors [16–18]. However, the molecular nature of alternative factors and how they interact with a functional RAG-PC is largely unknown.

To address these issues, we focus on severe combined immunodeficient (scid) mice that are defective in DNA-PKcs and are known to have leaky recombination activity *via* a DNA-PK-independent pathway [19,20]. We derived recombination inducible cell lines from scid mice, designated as scid-ts, by transformation of scid progenitor B cells with temperature sensitive Ableson Murine leukemia virus (Abl-MuLV). These cells display conditional resolution of newly induced recombination ends. Specifically, they can be induced by incubation at 39°C, i.e., the non-permissive temperature, to initiate recombination cleavage, but form very few joining products. A significant amount of coding joints and signal joints are made after these cells are returned to the permissive temperature, 33°C [21]. Thus, the alternative-NHEJ pathway could be readily induced in these cells *via* experimental manipulation, which offers a model to further explore molecular components and processes of the alternative NHEJ pathway. Our observation of a correlation between the level of CJs with the level of opened CEs, and an inverse correlation between end resolution and *RAG1/2* expression (Supplementary Fig. 1A) agrees with the idea that the *RAG1/2*-mediated PC may impede final end resolution [22–24]. Then, the disassembly of the PC complex, probably occurring at the permissive temperature in our scid-ts cells, may render the ends accessible to resolution machinery independently of DNA-PKcs. As such, we hypothesize that the accessibility of recombination ends in scid cells exerts an influence on the ability of non-classical NHEJ factors to recognize and execute joining of these ends in the absence of DNA-PKcs.

To directly test this hypothesis, we developed the recombination end protection assay (REPA) to monitor the accessibility of recombination ends to a processing enzyme, exonuclease-V

(Exo-V), which degrades double-strand DNA (ds-DNA) and single strand DNA (ss-DNA) with free DNA ends [25]. Here, we focused on the analysis of opened coding ends and signal ends, because of their technical feasibility. In particular, we examined whether these ends are free DNA breaks or protected by protein complexes. By analyzing susceptibility of recombination ends to Exo-V, we compared the accessibility of these ends in scid and wild type cells, and provide evidence for a correlation of opened CE accessibility with CJ formation in scid, but not wild type cells. Thus, our novel assay allows us to monitor recombination induced chromosomal breaks for their availability to end resolution machinery, offering important insight into the alternative pathway for end resolution in scid cells.

2. Materials and Methods

2.1. Mice and cell lines

Scid and wild-type Balb/c mice were bred and maintained as previously described [26]. Thymocytes from scid homozygous newborn (1–3 days old) mice were pooled (6–8 mice) to obtain 10 million cells. Wild-type thymocytes were harvested from Balb/c mice. The s/+ts and scid-ts cell lines were derived from scid and s/+ mice, respectively [26]. RAG2^{-/-} Abl-MuLV cell line, 63–12, was kindly provided by Dr. F. Alt (Harvard Medical School, Boston, MA) via Dr. M. Schlissel (University of California, Berkeley, Berkeley, CA). Temperature-sensitive cell lines were maintained at 33°C, and induced to undergo immunoglobulin light-chain gene rearrangement by incubation at 39°C for 48 hours. Cells returned to 33°C after induction were incubated for 24 hours before harvesting.

2.2. Recombination End Protection Assay

This assay is designed to test the susceptibility of endogenously produced broken DNA ends to exonuclease-V (Exo-V). While free DNA ends are expected to be susceptible to the Exo-V mediated digestion the ends bound by proteins or protein complex may be resistant to this enzyme treatment. Although hairpin CEs exist in large abundance in scid lymphocytes, it is technically difficult to examine their accessibility in intact nuclei as the physiological environment of the isolated nuclei is not compatible with enzymatic conditions necessary for hairpin processing enzymes, such as Mung Bean Nuclease (MBN). Therefore, we primarily focus on the analysis of opened CEs and SEs. Nuclei were isolated from inducible cell lines or primary thymocytes by lysing cells in hypotonic buffer (50mM Hepes, pH 7.5, 10mM KCl, 10mM MgCl₂, 1mM dithiothreitol) containing 0.1% IGEPAL and protease inhibitors (Sigma-Aldrich, St. Louis, MO). The released nuclei were collected by centrifugation at 3000 RPM for 1 min. 4–5 million scid nuclei and 10 million s/+ nuclei were resuspended in 100 µl and 200 µl of Exo-V digestion buffer, respectively. Isolated nuclei were divided among equivalent sample volumes of 50 µl, including buffer control at 4°C, buffer control at 37°C (in the presence or absence of dNTP), and buffer plus Exo-V (USB scientific, Cleveland, OH), at 7 or 15 units/50 µl. For inhibition experiments, the nuclei were first incubated with inhibitors [30 µM of the ATM inhibitor, KU 55933 (Calbiochem CAS 587871-26-9) and 30µM of the DNA-PK inhibitor NU 7026 (Sigma-Aldrich, CAS 154447-35-5), or 0.25mM of ATP-γ-S (Sigma-Aldrich, CAS 93839-89-5) on ice for 30 min before their incubation at 37°C. Stock solutions for the ATM and DNA-PKcs inhibitors were prepared in DMSO and a vehicle control sample was prepared at 0.1% in the final reaction volume. ATP-γ-S was prepared as a stock solution in H₂O. In some experiments, purified DNA molecules embedded in agarose-plugs were also included for the susceptibility of pre-existing DNA breaks to Exo-V-mediated degradation. Digestions were carried out at 37°C for 1 hour followed by the addition of 50 µl of molten 1.2% low melt agarose (SeaKem) before deproteinization with proteinase K (Roche, Indianapolis, IN).

2.3. Ligation-Mediated PCR (LM-PCR) and Southern Blotting

Recombination ends were detected by modified LM-PCR followed by Southern blot analysis, as described previously [27,28]. To examine broken staggered ends, purified DNA molecules embedded in agarose-plugs were treated with T4 DNA polymerase and then ligated to an artificial linker by T4 DNA ligase. To reveal hairpin ends, the plugs were first treated with mung bean nuclease (NEB), followed by T4 DNA polymerase, and then ligated to the linker. One 20th of ligated DNA plugs (equivalent to 200–250,000 cells) were amplified for 31 cycles to reveal coding and signal ends. Similar amounts of DNA were also amplified for CJs, SJs and germline control (GL) or actin. Given the abundance of joined products in the cells, CJs and SJs were amplified for 26 cycles whereas input control, actin or GL was amplified for 21–22 cycles. A semi-quantitative PCR reaction was also performed, i.e., several dilutions of ligated DNA were subjected to PCR to ensure relative linearity of PCR amplification (supplementary Fig. 2A). The intensity of PCR bands representing recombination ends and products was quantified using a Phosphorimager and analyzed by Image Quant software (Molecular Dynamics). The quantification of each band was adjusted to the input DNA control, i.e., actin (or GL), then divided by the control-end (i.e., the ends present in the nuclei incubated at 4°C or 37°C) to its actin ratio. For example, the calculation for CEs was $\text{sample}-(\text{CEs}/\text{actin}) \div \text{control}-(\text{CEs}/\text{actin})$. For Fig. 1, the end input (i.e., C) was arbitrarily set at 100% as a reference for the samples under various treatments. For the other figures, the intensity of the ends from the nuclei reaction at 37°C was used as a reference.

4. Results

4.1. Temperature-dependent regulation of end accessibility in scid-ts cells

Although scid cells are known to accumulate a significant amount of hairpin CEs, they also make opened CEs, presumably *via* a DNA-PKcs-independent pathway [21,29]. The correlation observed in scid-ts cells between the level of opened CEs with the level of CJs (Supplementary Fig. 1A) suggest that the opened CEs are accessible and available for their final resolution by an alternative pathway, and hairpin CEs might have already been opened by the alternative pathway. Thus, we chose to analyze the accessibility of opened CEs to determine whether these ends are free or bound by protein complexes. In addition, the status of hairpin CEs could also be inferred from the accessibility of opened CEs. To directly examine the accessibility of CEs (which are opened CEs, unless specified) and SEs, we developed REPA to measure the susceptibility of broken ends in intact nuclei to a processing enzyme, Exo-V. This enzyme was found permeable to the isolated nuclei as it could degrade chromosomal DNA breaks generated by micrococcal nuclease (Supplementary Fig. 1B). Fig. 1A illustrates the experimental scheme. Specifically, isolated nuclei were subjected to Exo-V digestion and then purified DNA was analyzed for the integrity of remaining recombination ends by ligation-mediated PCR (LM-PCR). The recombination ends, if bound and protected by protein complexes, would be resistant to Exo-V digestion whereas free ends would be susceptible. The higher the intensity of PCR products that is detected, the better protection offered to those ends by end-binding complexes.

If the conditional resolution of CEs observed in scid-ts cells, i.e., low at 39°C and high during the down-shift from 39 to 33°C (39–33), is attributed to the accessibility of opened CEs, we expect to see a fluctuation in the accessibility of these CEs following a pattern similar to conditional end resolution. To test this, we compared the susceptibility of these ends to nuclease treatment in nuclei isolated from cells under these two conditions, 39°C and 39–33.

Representative experiments shown in Fig. 1B and 1C analyzed recombination ends made at λ and κ loci, respectively, with the quantitative comparison given in Fig. 1D. The nuclei without Exo-V treatment serve as a positive control (Fig. 1B, lane 1 and 4), which reflect the total input of recombination ends in the sample. In agreement with our previous reports, more opened

CEs are present in cells at 39–33 than in cells at 39°C, implicating accessibility of hairpin ends to nicking during this phase by a DNA-PKcs-independent pathway [26,28]. The susceptibility of these opened ends to Exo-V was evident in purified DNA samples, as no LM-PCR products of CE or SE amplifications were detected (Fig. 1B, lane 2 and 5). CEs present in nuclei, however, could still be recovered after Exo-V treatment, indicating protection of the ends (Fig. 1B, lane 3 and 6). The level of protection at CEs differs depending on the recombination conditions, high at 39°C and low during 39–33, as shown in Fig. 1C (compare lane 3 to lane 6). Thus, as expected, the accessibility of CEs seems to follow the conditional pattern of end resolution, in that more accessibility of CEs favors CJ formation. The lesser accessibility of CEs at 39°C suggests that these ends may still be held by a RAG1/2-containing PC, preventing access to end resolution machinery. Upon returning the cells to 33°C, these ends may then be liberated from, or become less associated with, end binding complexes. PC dissolution may not only confer access to joining factors, but to nucleases as well, leading to nucleotide deletion at the junction [30].

Scid-ts samples were also analyzed for SE integrity after enzymatic modification. Unlike the finding at the CEs, significant amounts of SEs remain after treatment of nuclei with Exo-V, indicating they are protected from nuclease digestion. Furthermore, this protection was observed irrespective of prevailing culture conditions in the cell (i.e. 39 or 39–33; Fig. 1B, compare lane 1 and 4 to 3 and 6). Thus, CEs and SEs made at the λ -locus exhibit different end accessibility to the enzymatic treatment. A similar finding was also observed at recombination ends derived from the κ -locus (Fig. 1C), in which the PCR primers used allowed for amplification of SEs made at both J κ 1 and J κ 2 loci. These findings suggest that SEs, but not CEs, may be bound by protein complexes throughout the recombination process at both cleavage and resolution phases. Interestingly, while the accessibility of CEs is correlated with the resolution of CEs into CJs, the relative inaccessibility of SEs, especially during 39–33, does not seem to impede the resolution of these ends since a significant amount of SJs were found during this condition [28]. Potentially, the formation of SJs proceeds within a protein complex, highlighting differing end-accessibility requirements for SE and CE resolution. Thus, in line with previously reported observations of asymmetric resolution of CEs vs. SEs [30–32], the observed differences in end protection may also provide an explanation for the relatively intact SJs and deleted CJs in scid cells [30].

4.2. Protection of recombination ends in s/+ts cells

We inferred from the above findings that both coding and signal ends in wild-type cells (i.e., s/+ts) must be highly protected throughout the recombination process since SJs and CJs exhibit high joining fidelity. To directly address this question, we used REPA to examine the accessibility of SEs and CEs in a temperature sensitive wild-type cell line, s/+ts. Initial attempts were confounding in that higher levels of CEs were detected in the nuclei treated with Exo-V as compared to the input CEs present in the cells (data not shown). We thus speculated that the elevated CEs might have been generated during the incubation accompanying Exo-V treatment. Thus, to analyze these ends generated both *in vivo* (i.e., from the cells) and *ex vivo* (i.e., from the isolated nuclei during an *in vitro* incubation), we included additional controls. Nuclei prepared from s/+ cells at 39°C and 39–33 were resuspended in digestion buffer and subjected to three different experimental conditions, 1) kept at 4°C as a control for input DNA ends (4); 2) incubated at 37°C without Exo-V (37); and 3) incubated at 37°C in the presence of Exo-V (E7). The scid-ts nuclei were included for comparison (Fig. 2A, lane 7–9).

Similar to our previous studies as well as others [26,28,33,34], the level of input CEs in s/+ cells, i.e., the 4°C-control sample, was very low, even though SEs were readily detectable in these cells (Fig. 2A, lane 1 and 4). LM-PCR amplification with several diluted DNA samples further confirms this point (Supplementary Fig. 2A). We were very much intrigued by the

increased level of CE's present in the nuclei after incubation at 37°C. These CE's are likely generated *in situ* during the incubation at 37°C since the input nuclei, i.e., sample kept at 4°C, showed no or very low levels of such ends (Fig. 2A, compare lane 1 and 4 to 2 and 5). Opened CE's detected under both conditions (i.e., 4°C and 37°C) appear to be staggered as they could not be amplified without prior treatment with T4 DNA polymerase (Supplementary Fig. 2B), and were not the result of opening by non-specific nucleases since they could not be recovered from the RAG2^{-/-} nuclei under the same incubation (Supplementary Fig. 2C). These opened CE's could have been generated from RAG1/2-mediated *in situ* cleavage followed by end opening through action of Artemis/DNA-PKcs, Mre11/Rad50/Nbs1 or other unidentified nucleases. Regardless of how they were produced, these ends were resistant to Exo-V digestion, as the enzyme treatment caused no reduction in CE's of nuclei isolated from s/+ts cells cultured at 39°C (Fig. 2A, lane 2 and 3). Although some reduction in the level of CE's was observed in the s/+ts nuclei of down-shifted cells, i.e., 39–33 (Fig. 2A, lane 5 and 6), the level of protected CE's was still much higher than that of scid-ts nuclei under the same conditions (Fig. 2A, comparing lane 5 and 6 to lane 8 and 9). Figure 2B shows a quantitative comparison of the ends made in s/+ts nuclei, demonstrating *in situ* generation of CE's upon nuclei incubation at 37°C, as well as the stringent protection of both SE's and CE's.

An increased level of opened CE's was repetitively observed upon incubation of s/+ts nuclei at 37°C (Fig. 2, Fig. 3A and Supplementary Fig. 2). Although it is difficult to estimate actual CE levels in comparison to those made endogenously by intact cells, an elevation of these ends after an *in vitro* nuclei culture suggests their generation *in situ* (Fig. 2A, compare lane 2 and 5 to lane 1 and 4). Furthermore, these newly generated opened CE's appear inaccessible to both Exo-V and end resolution machinery since no CJs or SJs were accumulated during this time (Fig. 3A, compare lane 5 to 6, and 7 to 8 for the levels of CE's and CJs). The addition of dNTPs fails to increase the formation of CJs and SJs (unpublished observation). Thus, we argue that while the *in vitro* nuclei reaction allows revelation of a snapshot of production and accumulation of CE's at the time of nuclei isolation, it is incompatible with final end resolution. Moreover, this method offers an opportunity to follow the fate of CE's generated from the endogenous Ig gene loci. On the other hand, tracking these ends would be impossible within whole cells, as recombination proceeds too rapidly from cleavage to joining, leaving few CE's available for analyses.

The same cannot be said of scid nuclei as no *in situ* generation of opened CE's could be found in scid nuclei under the same reaction conditions (Fig. 3A, compare lane 1 to 2, and 3 to 4) despite having comparatively more pre-existing hairpin ends than s/+ts nuclei (Fig. 3B, lane 1–4 and lane 5–8). The hairpin ends were revealed by a modified LM-PCR, in which purified DNA samples, but not nuclei, were pre-treated with different doses of MBN followed by T4-DNA polymerase and then subjected to LM-PCR, as described previously [35]. Thus, during the *in vitro* nuclei-reaction, scid nuclei fail to convert hairpin CE's to opened CE's despite starting with a large population of available hairpins. Conversely, opened CE's are detected in s/+ts nuclei after an *in vitro* incubation though they have far fewer pre-existing hairpin CE's to start with. It is possible that generation of these CE's from s/+ts nuclei is dependent on functional DNA-PKcs. This speculation was supported by the finding that a nonhydrolyzable ATP analog, ATP-γ-S, significantly blocked the generation of CE's (Fig. 3C). While DNA-PKcs is known to process hairpin CE's, Ataxia Telangiectasia Mutated (ATM) protein has also been implicated in protecting chromosomal CE's [36]. To examine the role of these two enzymes in the *in situ* generation of opened CE's from s/+ts-nuclei, and possibly in the protection of these ends, we manipulated the nuclei reactions by including DNA-PKcs or ATM inhibitors, NU7026, and KU 55933, respectively. It is clear from Fig. 3D that compared to the vehicle control, DMSO (lane 2), there was virtually no detectable CE's in the nuclei reaction treated with the DNA-PKcs inhibitor (lane 4). The ATM inhibitor was found to cause reduction in both the level and the size of Jκ2-CE (Fig. 3D, lane 3). Taken together, these findings support

the notion that the *in situ* generation of opened CEs is at least dependent upon the enzymatic activity of DNA-PKcs and may require the activity of ATM to lend complimentary protection to the CEs recovered in this assay. Thus, DNA-PKcs and ATM may function together in protecting the integrity of newly generated opened CEs.

4.3. Comparison of end susceptibility between wild-type and scid thymocytes

By using the REPA assay, we have assessed the association of proteins with SEs and opened CEs under various experimental conditions. Three conclusions can be drawn from the above findings: 1) opened CEs could be generated *in situ* from wild-type, but not scid nuclei during their incubation at 37°C (Fig. 2 and 3); 2) opened CEs derived from wild-type cells are not readily accessible to Exo-V, and exhibit better protection than those in scid cells (Fig. 1 and Fig. 2); and 3) SEs are much better protected than opened CEs in the same scid cells (Fig. 1). To verify our approach and conclusion, we extended our analyses to primary thymocytes. We selected the TCR δ -DJ locus for analyzing the end accessibility of scid and wild-type thymocyte nuclei, since both J δ 1 coding and 3' D δ 2 signal ends can be revealed in the same LM-PCR amplification (Fig. 4A).

Consistent with previous reports [29,37,38], very few opened CEs were found in wild-type thymocytes, whereas a significant amount of these ends were detected in scid thymocytes (Fig. 4, lane 1 and 4). Like the observed *in situ* production of opened CEs in s/+ts nuclei, opened CEs were also generated from nuclei of wild-type thymocytes during their incubation at 37°C (Fig. 4A, lane 1 and 2). The amplified LM-PCR products from these CEs were cloned for sequence analysis. As shown in Table 1, seven D δ 2-CEs and three J δ 1-CEs display authentic CEs with relatively intact D δ 2 and/or J δ 1 gene segments, in which the perfect J δ 1-CE (i.e., clone J δ 1-1) was detected repetitively from thymocyte nuclei of two individual mice. Additionally, three non-standard (NS) ends were also recovered. Two were derived from the delta locus 87 or 164 bases 5' of J δ 1 locus while the other contained a rearranged V δ D δ J δ -joint, containing broken ends in the middle of the V α -region. It is possible that these ends resulted from non-RSS-dependent cleavage, reflecting the infidelity of end processing in isolated nuclei. Nonetheless, the majority of the CEs was authentic and appeared to be resistant to Exo-V digestion, implicating their association with protein complexes (Fig. 4C, lane 2 and 3). In contrast, the opened CEs in the nuclei of scid thymocytes were poorly protected. As demonstrated in Fig. 4B, these CE ends were not only sensitive to Exo-V digestion (lane 6), but also appear susceptible to endogenous nucleases present in the nuclei since the level of CEs at 37°C was lower than that of the control, i.e., the nuclei at 4°C (Fig. 4B, lane 4 and 5). On the other hand, a significant amount of intact 3'D δ 2-SEs was still retained after Exo-V treatment of nuclei isolated from both scid and wild type thymocytes (Fig. 4B, lane 4–6 and lane 1–3, top band). Thus, consistent with our finding in scid-ts cell lines, SEs generated from both wild-type and scid thymocytes were largely protected from enzymatic modification.

We can therefore conclude that opened CEs in primary scid thymocytes may be less, or unassociated with protective complexes making them available for alternative NHEJ resolution. CEs generated in the DNA-PKcs deficient background are more accessible to alternate end binding factors than their wild type counterparts, but may consequently be vulnerable to nucleases and aberrant joining common to scid end resolution. Taken together with the ts-cell line data, we confirm that opened CEs are better protected in wild-type cells than in scid cells while SEs are more resistant to Exo-V than CEs in both cell types.

Discussion

REPA offers a novel tool to examine the accessibility of newly generated chromosomal recombination breaks, and indirectly assess the stability of protein complexes formed at these breaks, especially those that participate in the generation and resolution of recombination ends.

While opening of hairpin CE occurs *en masse* after induction and before joining, there remain some open CEs at the transition from RAG to NHEJ, which can be influenced by DNA end management. We focused on the protection and accessibility of open CEs due to technical feasibility, and are less concerned with the hairpin opening process. The presence and stability of an end-binding complex relates to its ability to protect associated DNA ends from digestion by exonucleases. The fact that recombination ends are relatively inaccessible in wild type cells argues that these ends are likely held within protein complexes throughout the recombination reaction. Conversely, open CEs in scid-ts cells display conditional accessibility, i.e., less accessible during RAG1/2-mediated cleavage at 39°C, and more accessible after cells are returned from 39 to 33°C (Fig. 1), where RAG proteins are down-regulated and rearranged CJs are formed (Supplementary Fig. 1A). Scid CE resolution in this system is presumably mediated by a DNA-PK-independent alternative pathway, a back-up system that is apparent in other NHEJ mutant cells [39]. Several well executed investigations have identified RAG1/2 as a hub, linking V(D)J recombination ends to their principle end joining program, NHEJ (14, 39). RAG mutations affect the stability of PCs, making recombination ends available to alternate repair [14,15]. These studies argue that pre-mature release of recombination ends from the PC formed by RAG mutants may render the ends accessible to alternative NHEJ pathways for end joining. In line with this argument, our findings suggest that in the presence of normal RAG1/2, the alternative pathway may only function on those CEs that escape from the PC, pinning the ability to resolve these ends on the destabilization of the PC. This alternative event could be influenced by the prevailing cellular RAG levels or other, less well defined processes. Thus, RAG generated CEs fall through the normal RAG-NHEJ recombination axis, either due to instability of the PC or defective NHEJ machinery. Our scid-ts system approximates this by artificially manipulating the stability of the RAG complex formed at CEs in a DNA-PKcs deficient, non-classical NHEJ environment. Therefore, we can “force” alternative end joining by modulating RAG levels and shunting CEs from a stable PC complex to a poorly protective one as revealed by the Exo-V accessibility analysis. For scid thymocytes, their apparent CE accessibility reflects poor stability or dissociation of the PC, which likely accounts for readily detected leaky recombination observed in scid mice, as well as in other NHEJ mutant models [40–42].

SEs are processed very differently from CEs. We show here that SEs are less accessible than CEs during the conditions of both the non-permissive and permissive temperatures (Fig. 1), implicating better protection of SEs by protein complexes. Thus, scid SEs are likely held within protein complexes throughout cleavage and resolution phases, similar to the pattern displayed by their wild type counterpart. The observed inaccessibility of SEs in both wild type and scid cells is consistent with the reported high binding affinity of RAG1/2 to SEs shown in a cell-free assay [6,43], as well as demonstrated by chromatin-immunoprecipitation (CHIP) using anti-RAG antibodies [44]. Interestingly, the persistent association of SEs with the protein complex does not seem to be correlated with conditional resolution of these SEs as they could be resolved under relatively inaccessible conditions (Fig. 1). These data suggest that the resolution of SEs may not rely on their free status, but proceed within synaptic complexes, including RAG1/2, Ku70/80 or XLF/XRCC4/LigIV, which can be recruited to DNA ends independent of DNA-PKcs [45,46].

The resolution of intermediate DNA ends in wild type cells is far more compartmentalized and tightly regulated, as revealed by minute amounts of detectable unresolved ends. This apparent lack of CEs is indicative of rapid end processing coupled with prompted end joining [33–35]. Under our *in vitro* nuclei reaction, the rapid CE resolution process found in wild-type cells is somewhat disrupted. This allows for the generation and accumulation of some opened CEs to be analyzed using REPA (Fig. 2, 3 and 4). The majority of these CEs are of a staggered structure, resembling those made in whole cells (supplementary Fig. 2). The authentic nature of TCR-DJ δ CEs recovered from the nuclei reaction of wild-type thymocytes suggests that nuclei retain

the use of resolution machinery to process hairpin ends derived from chromosomal recombination substrates *ex vivo*. We argue DNA-PKcs/Artemis may be involved in processing hairpin ends within nuclei, since scid nuclei isolated from cells undergoing recombination cleavage (i.e. cells cultured at 39) possess a significant amount of hairpin ends (Fig. 3B lane 2–4), but were not converted to opened CE (Fig. 3A, lane 1 and 2). Furthermore, the production of opened CE in wild-type nuclei is abrogated by a DNA-PKcs inhibitor (Fig. 3D), indicating the function of DNA-PKcs in the *in situ* generation of opened CE.

These newly generated opened CE appear to be protected in wild-type nuclei presumably by a more stable PC complex than scid nuclei, inferred from their resistance to exonuclease treatment (Fig. 2 and Fig. 4). DNA-PK has long been speculated to function as a scaffold for modulating and recruiting end joining factors to the post-cleavage complex. It was demonstrated *in vitro* that DNA-PKcs is able to bind DNA termini and block them from degradation and ligation just as Ku70/80 is thought to directly interact with RAG1 [47]. In addition, ATM has also been reported to maintain CE in PC complexes [48]. In support of this study, we found a reduction in the amount and relative size of J κ 2-CEs recovered from the s/+ nuclei reaction treated with an ATM inhibitor, thus confirming the role of ATM in maintaining CE integrity. It is possible that DNA-PKcs may cooperate with ATM, forming a large scaffolding complex that functions to retain CE for proper joining. It remains to be determined how DNA-PKcs protects these ends, either through direct interaction or more indirectly by stimulating end protection *via* ATM modification.

Supplementary Material

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Fig. 1. Accessibility of recombination ends in intact nuclei of scid cells by REPA

A. Diagrammatic representation of generic recombination substrates that are protected (Left panel) or degraded (right panel). Dashed lines indicate linkers and arrows denote PCR primers. The diamond and triangle represent protein complexes holding to the ends. **B.** The accessibility of recombination coding and signal ends at Vλ-locus (denoted as Vλ-CE and Vλ-SE, respectively) was assessed by their sensitivity to 7 units exonuclease-V (E7), in which nuclei were prepared from cells either cultured at 39°C or down-shifted from 39°C to 33°C (39–33). The nuclei kept at 4°C serve as input end control (denoted as C). E represents exonuclease-V treatment of purified DNA as a control for the susceptibility of the ends to this enzymatic treatment. **C.** REPA analysis of recombination ends derived from the Jκ-locus. The primers

used allow detection of two Jk signal ends (Jk1-SE and Jk2-SE). E7 and E15 are nuclei samples digested with 7 or 15 units/50µl of Exo-V. **D.** Quantitative analyses of PCR products amplified from recombination ends. The PCR bands were quantified using Image Quant software. C is arbitrarily set at 100%, representing input DNA ends. Samples E and E7 are expressed as percentages of C, i.e., % protection against the input. The bar graph summarizes 3 independent experiments, presented as an average of relative protection with standard errors.

Fig. 2. Comparison of recombination end accessibility in wild-type (s/+ts) cell lines

A. Vλ coding and signal ends were analyzed by REPA. 4 and 37 are control (undigested) nuclei incubated at 4°C and 37°C respectively. E7 are nuclei treated with 7 units of exonuclease-V. The scid-ts nuclei isolated from the cells cultured at 39–33 were included for end comparison. **B.** Quantification of LM-PCR products amplified from Vλ CEs and SEs of induced s/+ts cell lines. The CEs derived from 37°C of each cellular condition is arbitrarily set as 100% for the comparison. See Fig. 1D for details.

Fig. 3. Analyses of *in situ* generation of opened CEs

A. Examination of recombination ends and joining products generated during the nuclei reaction at 37°C. Nuclei isolated from both scid (s/s-ts) and wild-type (s/+ts) cells, cultured at 39°C or down-shifted from 39°C to 33°C (i.e. 39–33), were either kept at 4°C or incubated at 37°C for one hour. The *in situ* production of recombination ends (Vλ-CE and Vλ-SE) was determined by an increase of amplified products from the background level at 4°C to the one at 37°C. **B.** Opened and hairpin coding ends were analyzed by a modified LM-PCR. The DNA samples in lane 1 and 5 were treated with T4 DNA polymerase followed by LM-PCR, in which their LM-PCR products were amplified from opened coding ends. The rest of the samples of both scid-ts and s/+ts cells (i.e., lane 2–4 and 6–8) were treated with different doses of

mungbean nuclease (MBN) (5, 10 or 15 U) followed by T4 DNA polymerase before being subjected to LM-PCR. The increased PCR products observed in these samples over the T4-controls reflect the presence of hairpin ends. The analysis of SEs serves as a control for recombination initiation. C. Inhibition of *in situ* production of opened CE by an ATP inhibitor. The nuclei of s/+ts cells cultured at 39°C were incubated at 37°C in the presence of ATP- γ -S (γ -S) before being assessed by REPA, in which nuclei kept at 4°C and 37°C were included as controls. D. Effect of protein kinase inhibitors on *in situ* production of CE from s/+ts nuclei. Nuclei isolated from s/+ts cells cultured at 39°C were pre-treated with ATM inhibitor (A(i)), DNA-PKcs inhibitor (D(i)) or DMSO (DM, at the final concentration of 0.1%) before their incubation at 37°C for end production. SEs and CE derived from Jk1 and Jk2 loci were analyzed by LM-PCR.

Fig. 4. Accessibility of recombination ends in primary thymocytes of scid and wild-type mice
A. A digramatic representation of the TCR D δ 2 and J δ 1 germline (GL) structure and potential recombination intermediates made at this locus. Rectangles indicate coding regions and black and white triangles represent 12 and 23-RSS, respectively. Detection of recombination CEs and SEs were possible by linker (L) ligation to these end structures and subsequent PCR amplification (small arrows indicate PCR primers). The distance of the gene segments is not depicted in proportion. **B.** LM-PCR followed by Southern blot analysis of D δ 2-SEs and J δ 1/D δ 2J δ 1 CEs from wild-type (wt) and scid thymocytes. Primary thymocytes were harvested, and nuclei suspensions were prepared. Nuclei were subjected to REPA analysis as shown previously and including nuclei incubated at 4°C and 37°C as controls (see Materials and

Methods). **C.** Quantification of recovered coding and signal ends follows the similar procedure described in Fig. 2B. The bands representing D δ 2 SEs and J δ 1/D δ 2J δ 1 CEs indicated by the arrows shown in **B** were quantified.

Table 1

Sequence analysis of opened coding ends recovered from nuclei of wild-type thymocytes

Clone	N/P-insertion ^a	Dδ2-segment (N/P ^b)	Jδ1-segment
Germline		ATCGGAGGGATACGAG	CTACCGACAAACTCGTC
Jδ1-1 ^c			CTACCGACAAACTCGTC
Jδ1-2	GATGGCT		CTACCGACAAACTCGTC
Jδ1-3	TGTCGGTAG		CTACCGACAAACTCGTC
Dδ2-1	GC	ATCGGAGGGATACGAG	CTACCGACAAACTCGTC
Dδ2-2	CAT	GGATACGAG	CTACCGACAAACTCGTC
Dδ2-3		GGATAGA	TACCGACAAACTCGTC
Dδ2-4	CATCCT	ATCGGAGGGA	TACCGACAAACTCGTC
Dδ2-5	CATCAT	ATCGGAGGGATA ATTG	CTACCGACAAACTCGTC
Dδ2-6	GGCCCTCAGC	CGGAGGGATACGAG	CTACCGACAAACTCGTC
Dδ2-7	TCTCTGCTATGAGTGGC	ATCGGAGGGA	TACCGACAAACTCGTC
NS-1 ^d	87bps upstream of Jδ1		CTACCGACAAACTCGTC
NS-2 ^d	164bps upstream of Jδ1		CTACCGACAAACTCGTC
NS -3 ^d	TCR-Vα-10 (256–334) ^e	ATCGGAGGGATACGAGG	CTACCGACAAACTCGTC

^a: nucleotide insertion at the detected coding ends (Jδ1 or 5'Dδ2), of which the bold letters with italics are P-additions.

^b: letter in bold stands for nucleotide addition at the junction of DJ-coding joints between Dδ2 and Jδ1, in which the ones with italic are P-additions.

^c: observed in two individual mice

^d: non-standard (NS) ends recovered

^e: part of TCR-Vα-10 sequences derived from position 256 to 334.