

## VDJ Recombination: Artemis and Its In Vivo Role in Hairpin Opening

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Artemis is the newest player in VDJ recombination and double strand break repair. First identified in radiation-sensitive and immune-deficient patients, it was recently shown to interact with DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and have nuclease activity, becoming the most popular candidate for the opening of hairpin coding ends. Reports presented in this issue (1) and in the December issue of *Molecular Cell* (2) address the role of Artemis in vivo by studying the effect of its deletion on the generation of intermediates and products of VDJ recombination in mouse ES cells, MEFs, and thymocytes. In all three systems analyzed, a defect in coding joint formation was observed, which was highly dependent on the cellular background and included inefficient and partially defective opening of hairpin coding ends. In addition, Artemis-deficient ES cells and Artemis-deficient MEFs show spontaneous chromosomal abnormalities, including telomere fusions, indicating that Artemis is also required for maintenance of genomic stability. This commentary will focus on the results regarding the role of Artemis in VDJ recombination.

VDJ recombination, also known as antigen receptor gene rearrangement, is the process that assembles the variable domain of immunoglobulin and TCR genes. The hallmark of this reaction is the production of a large repertoire of antigen receptors with different specificities, a characteristic that is essential to the normal functioning of the immune system.

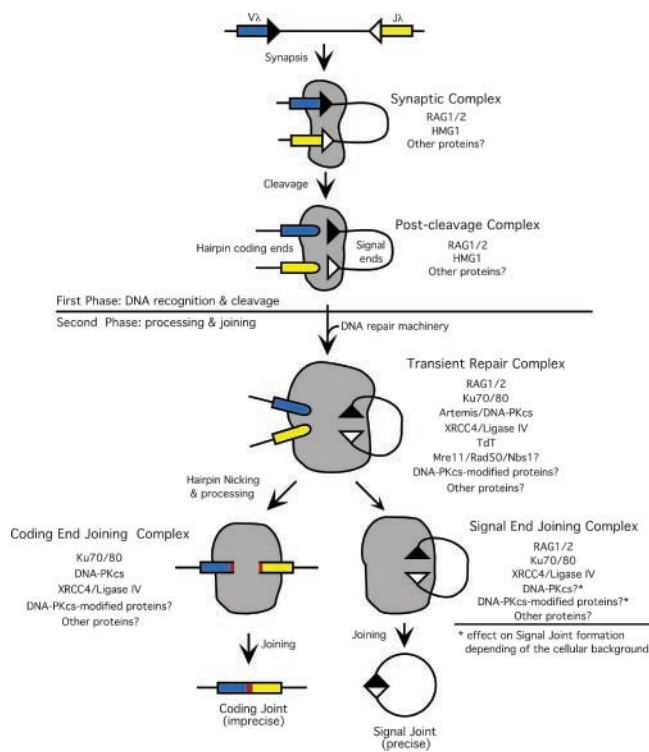
VDJ recombination is directed to the immunoglobulin and TCR loci by highly conserved recombination signal sequences (RSSs) comprised of a heptamer and a nonamer motif with an intervening 12- or 23-bp spacer. Efficient recombination in vivo occurs almost exclusively between RSSs with different spacers, which is referred to as the 12/23 rule (3). Several proteins that mediate VDJ recombination in vivo have been identified (4). The lymphoid-specific components of the recombination machinery are RAG-1, RAG-2, and terminal deoxynucleotidyl transferase (TdT). RAG-1 and RAG-2 together constitute the recombinase. TdT, although not essential for catalysis, plays an important

role increasing diversity by mediating the incorporation of nontemplate-dependent nucleotides. The nonlymphoid-restricted components identified so far are DNA-PKcs, Ku70, Ku80, XRCC4, ligase 4, and the most recently identified Artemis. All these proteins are involved in DNA double strand break repair as well as VDJ recombination. These nonlymphoid-specific components are likely to participate in the processing and joining steps of VDJ recombination, but their specific architectural or catalytic roles in the reaction remain largely unclear. Two other nonlymphoid-specific components, HMG1 and HMG2, have been implicated in VDJ recombination. In vitro experiments showed that these proteins increase the efficiency of cleavage by RAGs (5, 6). The role of HMG1 and HMG2 in VDJ recombination in vivo remains to be investigated.

During the initial stages of the reaction, RAG-1/RAG-2 form a complex with the RSS, which is in part stabilized by the interactions between the nonamer binding domain of RAG-1 and the nonamer motif. Bridging of 12 and 23 RSSs in a synaptic complex is critical for DNA cleavage and it seems to be facilitated by the DNA bending proteins HMG1 and HMG2 as well as other yet unidentified cellular factors (Fig. 1). Within the synaptic complex, RAG-1/RAG-2 efficiently introduce a nick at each RSS via a hydrolysis reaction at the heptamer/coding flank border, generating a 3' hydroxyl end. Subsequently, a transesterification reaction that resembles the mechanism of transpositional recombination creates a double strand break as the free 3' hydroxyl of the nicked strand is used in a nucleophilic attack on the opposing strand generating a covalently sealed hairpin intermediate, known as the hairpin coding end (5, 6). Opening and processing of this hairpin intermediate is a critical step in the generation of junctional diversity and has been the topic of intensive analysis in the last few years.

In vitro experiments demonstrated that after RSS cleavage, RAG-1/RAG-2 and HMG1 proteins remain bound in a complex with the resulting 5' phosphorylated blunt signal end and the hairpin coding end in what is known as the postcleavage complex (7). At this stage, the generation of junctional diversity is initiated by opening of hairpin coding ends leading to the formation of open double strand ends, which may contain single stranded overhangs. Also at this stage, the open coding ends are potential sub-

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**Figure 1.** Schematic representation of protein–DNA complexes in VDJ recombination. Blue and yellow rectangles represent the coding segments to be recombined (exemplified as V $\lambda$  and J $\lambda$ ). The 12-RSS and 23-RSS are represented as white and black triangles, respectively. The red rectangle represents the newly generated nucleotide sequence at coding junction and the gray represents protein complexes.

strates for nontemplate nucleotide additions by TdT, polymerase extension leading to palindromic nucleotides, nuclease attack generating small coding element deletions, and microhomology-directed nonhomologous end joining (NHEJ) via annealing of short complementary stretches of the overhangs. These coding end intermediates, arising at this late stage of VDJ recombination, have common branched structures characterized by duplex/single strand junctions lacking consensus binding motifs (8). Many of these recombination intermediates could be processed by the nuclease activity of Artemis, RAG-1/RAG-2, or the Mre11/Rad50/NBS1 (MRN) complex. Evidence supporting the participation of these protein complexes in hairpin coding end opening and processing will be further discussed.

Ultimately, the VDJ recombination reaction is concluded with the joining of the processed coding ends and the unprocessed signal ends by the ubiquitously expressed double strand break repair proteins. Although it is not clearly known when the double strand break repair factors are incorporated in the recombination complex, it is plausible that they coexist with RAG-1/RAG-2 during the opening and processing of the coding ends as suggested in the scheme presented in Fig. 1. Furthermore, evidence accumulated so far suggests that coding and signal joints are formed independently (4), which argues for the existence

of different protein–DNA complexes during the later stages of VDJ recombination.

*Is Artemis the In Vivo Hairpin Opening Nuclease?* Identification and cloning of Artemis were accomplished very recently as the result of the systematic study of radiosensitive and immunodeficient (RS-SCID) patients by the de Villartay laboratory. Transformed fibroblasts from these patients were shown to have defects in VDJ recombination at the level of coding joint formation in addition to increased cellular radiosensitivity (9). The disease-related locus was mapped to chromosome 10 and subsequently cloned (10, 11). The same gene was shown to be defective in a group of Athabascan immunodeficient patients (12). Protein sequence analysis of Artemis suggested that it belongs to the metallo- $\beta$  lactamase superfamily (11). This classification was further supported by a comprehensive sequence analysis (13). Based on its exclusive effect in coding joint formation and not in signal joint formation, as well as its homology to proteins with hydrolase activity, Artemis was proposed to participate in opening the hairpin coding ends (11). This prediction was supported by in vitro studies performed in M. Lieber's laboratory (14), which showed that Artemis formed a complex with DNA-PKcs and this complex possesses hairpin opening activity. These findings have been previously discussed elsewhere (15). Ma et al. (14) also showed data suggesting that Artemis represents an in vitro substrate of DNA-PKcs, however, these experiments were done with a highly inactive NH<sub>2</sub>-terminal GST–Artemis fusion protein and sufficient controls were not provided to exclude the phosphorylation of the GST moiety. Thus, this result, although interesting, needs additional and more rigorous analysis.

The role of Artemis in hairpin opening is now further supported by a study presented in this issue. Rooney et al. (1) inactivated Artemis in murine ES cells by targeted mutation. As seen in fibroblasts derived from RS-SCID patients with a defect in the Artemis gene, Artemis-deficient ES cells were impaired in coding joint but not in signal joint formation. Although coding joint formation in Artemis-deficient ES cells was inefficient, coding joints were found and shown to have a unique structure. They frequently lacked deletions and displayed large junctional palindromes. In contrast, coding joints in Artemis-deficient MEFs showed very frequent and large deletions (2) similar to those found in DNA-PKcs-deficient MEFs and ES cells. The leaky phenotype of the Artemis-deficient mouse allowed the analysis of coding joints isolated from the TCR- $\beta$  locus. Surprisingly, D-J $\beta$  junctions were not significantly different from wild-type coding junctions (see supplemental Fig. 1 in reference 2), yet accumulation of hairpin coding end intermediates in these thymocytes was evident. However, as indicated by Rooney et al. (2), large deletions could not have been detected with the primer used in the analysis. In addition, a biased representation of coding joints may result after strong selection of successful rearrangements in vivo. Therefore, it will be very informative to study Artemis-deficient precursor

lymphocytes using exogenous recombination substrates. Analysis of coding joints isolated from these cells will help to determine if the normal coding joints isolated from Artemis-deficient thymocytes represent an artifact of selection and/or the PCR analysis, or if they reflect differences in the role and requirement of Artemis between different cell types.

In summary, a consistent phenotype in all types of Artemis-deficient cells analyzed is the low efficiency in opening of hairpin coding ends as reflected by accumulation of hairpin intermediates in Artemis-deficient thymocytes and low efficiency of coding joint formation in Artemis-deficient ES cells and MEFs. To explain the low efficiency of hairpin opening, Rooney et al. (2) suggested that coding joint formation and hairpin opening in the absence of Artemis are most likely formed by the classical NHEJ components, with other factors substituting for Artemis to open hairpin coding ends. The possible participation of RAG-1/RAG-2 and the MRN complex was also discussed as a nuclease activity that could function to open hairpin coding ends at a low level in the absence of Artemis. However, considering the results presented in the two papers previously discussed, as well as prior published data, it has become evident to consider RAG-1/RAG-2 and MRN complexes as true candidates for normal hairpin opening.

Besides its role in RSS recognition and DNA cleavage, the RAG-1/RAG-2 complex has also been proposed to participate in the postcleavage phase of VDJ recombination. After DNA cleavage, RAG proteins remain associated with coding and signal ends in the so-called postcleavage complex (7), which may help to organize processing and joining events (16). RAG proteins as well as DNA present in this complex may contribute to the recruitment of DNA repair factors (Fig. 1). Studies performed at the Schatz laboratory (17) provided evidence for an architectural role of RAG proteins in the second phase of VDJ recombination. Tsai et al. (17) reported a single mutation in RAG-1 (S723A or S723C) that renders the RAG-1/RAG-2 complex unable to form the postcleavage complex *in vitro*, and reduces the efficiency of coding and signal joint formation *in vivo*. In addition to this architectural role, RAG proteins have been shown to open hairpin coding ends *in vitro* using a 12/23-regulated cleavage reaction containing  $Mg^{2+}$  (18, 19) and process 3' flap structures (20). These activities may contribute to the processing of hairpin coding ends *in vivo*. Mutants of RAG-1 and RAG-2 have been isolated, which are active in DNA cleavage but impaired in coding joint formation *in vivo* and in opening of artificial hairpins *in vitro* (21, 22). In addition, Tn10 transposase, the closer relative of RAG-1/RAG-2 recombinase, was shown to catalyze hairpin formation and hairpin opening during the transposition reaction (23). Thus, hairpin opening and coding end processing activity may represent a critical function of RAG proteins in the context of the postcleavage complex and in VDJ recombination.

Similarly, evidence for the participation of the MRN

complex in hairpin opening has been published. The MRN complex efficiently opens hairpins *in vitro* in the presence of  $Mn^{2+}$  (24). Yet, hairpin opening by this complex has not been observed in  $Mg^{2+}$  conditions. Because 12/23 regulation has only been observed in  $Mg^{2+}$  conditions, it has been assumed that all the steps in VDJ recombination have the same cofactor requirement. This is an assumption without experimental support. In addition, in the context of the postcleavage complex, the MRN complex might be recruited and its properties and requirements might be modified to participate in hairpin opening. *In vivo* evidence for the hairpin opening activity of MRN complex comes from genetic studies in yeast, in which the MRN complex was shown to be required for the resolution of hairpins formed by inverted repeats during replication (25). A possible role of the MRN complex in VDJ recombination was further suggested when NBS1, one of the components of the MRN complex, was shown to colocalize with  $\gamma$ H2AX and TCR- $\alpha$  locus to sites that correlate with double strand breaks in T cell progenitors undergoing VDJ recombination (26). Therefore, it is plausible that MRN complex may have a direct role in coding end processing. Furthermore, MRN complex has been shown to increase the efficiency of ligation in a system using purified components that included the yeast homologues of XRCC4/ligase 4 and Ku70/Ku80 (27). It was also purified from HeLa cell extracts as an activity required for efficient ligation in a mammalian NHEJ system (28). These findings suggest that the MRN complex may also participate in the coding end and/or signal end ligation.

In summary, recent reports suggested the participation of Artemis/DNA-PKcs, RAG-1/RAG-2, and the MRN complex in the processing of hairpin coding ends. The participation of these machineries in the context of the postcleavage complex should be carefully investigated and the possibility that these complexes may participate together to generate highly diverse coding joints should also be considered.

Future experiments using point mutants of Artemis to separate the catalytic versus the architectural role of the protein will be very informative. One such mutant might be the one used by Ma et al. (14). The mutation (D165N, numbering according to reference 14), identified in a SCID patient, resides in the conserved SMN1 domain and the resulting mutant protein is unable to open hairpin substrates. Another interesting mutation (G111V, numbering according to reference 11) recently identified in a RS-SCID patient also resides in the SMN1 domain (29). VDJ recombination assays using fibroblasts obtained from this patient showed a deficiency in coding joint formation, a defect overcome by complementation with a wild-type Artemis-expressing vector. Therefore, it is of interest to determine the effect of these mutations on the accumulation of hairpin coding ends *in vivo*. On the other hand, it will be very interesting to investigate the effect of these mutant proteins on hairpin opening *in vitro* using the 12/23-regulated cleavage assay.

**Concluding Remarks.** This is an exciting moment in the field of VDJ recombination and DNA repair, as these two studies (1, 2) provide us with some answers and questions regarding the in vivo function of Artemis in these two processes. Regarding VDJ recombination, they leave us with a more intriguing question, which is what are the catalytic and architectural roles of Artemis/DNA-PKcs, RAG-1/RAG-2, and MRN complex during opening and processing of the hairpin coding ends? Thus, further experiments addressing this question will provide us with more insights into the processing of hairpin coding ends and the generation of junctional diversity.

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## References

- Rooney, S., F.W. Alt, D. Lombard, S. Whitlow, M. Eckersforff, J. Fleming, S.D. Fugmann, D.O. Ferguson, D. Schatz, and J. Sekiguchi. 2002. Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. *J. Exp. Med.* 197:553–565.
- Rooney, S., J. Sekiguchi, C. Zhu, H.-L. Cheng, J. Manis, S. Whitlow, J. DeVido, D. Foy, J. Chaudhuri, D. Lombard, et al. 2002. Leaky scid phenotype associated with defective V(D)J coding end processing in artemis-deficient mice. *Mol. Cell.* 10:1379–1390.
- Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature.* 302:575–581.
- Bassing, C.H., W. Swat, and F.W. Alt. 2002. The mechanism and regulation of chromosomal V(D)J recombination. *Cell.* 109:S45–S55.
- Fugmann, S.D., A.I. Lee, P.E. Shockett, I.J. Villey, and D.G. Schatz. 2000. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annu. Rev. Immunol.* 18:495–527.
- Gellert, M. 2002. V(D)J recombination: rag proteins, repair factors, and regulation. *Annu. Rev. Biochem.* 71:101–132.
- Hiom, K., and M. Gellert. 1998. Assembly of a 12/23 paired signal complex: a critical control point in V(D)J recombination. *Mol. Cell.* 1:1011–1019.
- Lewis, S.M. 1994. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. *Adv. Immunol.* 56:27–150.
- Nicolas, N., D. Moshous, M. Cavazzana-Calvo, D. Papadopoulos, R. de Chasseval, F. Le Deist, A. Fischer, and J.P. de Villartay. 1998. A human severe combined immunodeficiency (SCID) condition with increased sensitivity to ionizing radiations and impaired V(D)J rearrangements defines a new DNA recombination/repair deficiency. *J. Exp. Med.* 188:627–634.
- Moshous, D., L. Li, R. Chasseval, N. Philippe, N. Jabado, M.J. Cowan, A. Fischer, and J.P. de Villartay. 2000. A new gene involved in DNA double-strand break repair and V(D)J recombination is located on human chromosome 10p. *Hum. Mol. Genet.* 9:583–588.
- Moshous, D., I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, et al. 2001. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell.* 105:177–186.
- Li, L., D. Moshous, Y. Zhou, J. Wang, G. Xie, E. Salido, D. Hu, J.P. de Villartay, and M.J. Cowan. 2002. A founder mutation in Artemis, an SNM1-like protein, causes SCID in Athabaskan-speaking Native Americans. *J. Immunol.* 168:6323–6329.
- Callebaut, I., D. Moshous, J.P. Mornon, and J.P. de Villartay. 2002. Metallo-beta-lactamase fold within nucleic acids processing enzymes: the beta-CASP family. *Nucleic Acids Res.* 30:3592–3601.
- Ma, Y., U. Pannicke, K. Schwarz, and M.R. Lieber. 2002. Hairpin opening and overhang processing by an artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell.* 108:781–794.
- Schlissel, M.S. 2002. Does artemis end the hunt for the hairpin-opening activity in v(d)j recombination? *Cell.* 109:1–4.
- Sadofsky, M.J. 2001. The RAG proteins in V(D)J recombination: more than just a nuclease. *Nucleic Acids Res.* 29:1399–1409.
- Tsai, C.L., A.H. Drejer, and D.G. Schatz. 2002. Evidence of a critical architectural function for the RAG proteins in end processing, protection, and joining in V(D)J recombination. *Genes Dev.* 16:1934–1949.
- Besmer, E., J. Mansilla-Soto, S. Cassard, D.J. Sawchuk, G. Brown, M. Sadofsky, S.M. Lewis, M.C. Nussenzweig, and P. Cortes. 1998. Hairpin coding end opening is mediated by RAG1 and RAG2 proteins. *Mol. Cell.* 2:817–828.
- Shockett, P.E., and D.G. Schatz. 1999. DNA hairpin opening mediated by the RAG1 and RAG2 proteins. *Mol. Cell. Biol.* 19:4159–4166.
- Santagata, S., E. Besmer, A. Villa, F. Bozzi, J.S. Allingham, C. Sobacchi, D.B. Haniford, P. Vezzoni, M.C. Nussenzweig, Z.Q. Pan, et al. 1999. The RAG1/RAG2 complex constitutes a 3' flap endonuclease: implications for junctional diversity in V(D)J and transpositional recombination. *Mol. Cell.* 4:935–947.
- Qiu, J.X., S.B. Kale, H.Y. Schultz, and D.B. Roth. 2001. Separation-of-function mutants reveal critical roles for RAG2 in both the cleavage and joining steps of V(D)J recombination. *Mol. Cell.* 7:77–87.
- Schultz, H.Y., M.A. Landree, J.X. Qiu, S.B. Kale, and D.B. Roth. 2001. Joining-deficient RAG1 mutants block V(D)J recombination in vivo and hairpin opening in vitro. *Mol. Cell.* 7:65–75.
- Kennedy, A.K., A. Guhathakurta, N. Kleckner, and D.B. Haniford. 1998. Tn10 transposition via a DNA hairpin intermediate. *Cell.* 95:125–134.
- Paull, T.T., and M. Gellert. 1999. Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev.* 13:1276–1288.
- Lobachev, K.S., D.A. Gordenin, and M.A. Resnick. 2002. The Mre11 complex is required for repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements. *Cell.* 108:183–193.
- Chen, H.T., A. Bhandoola, M.J. Difilippantonio, J. Zhu, M.J. Brown, X. Tai, E.P. Rogakou, T.M. Brotz, W.M.

- Bonner, T. Ried, et al. 2000. Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX. *Science*. 290: 1962–1965.
27. Chen, L., K. Trujillo, W. Ramos, P. Sung, and A.E. Tomkinson. 2001. Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. *Mol. Cell*. 8:1105–1115.
28. Huang, J., and W.S. Dynan. 2002. Reconstitution of the mammalian DNA double-strand break end-joining reaction reveals a requirement for a Mre11/Rad50/NBS1-containing fraction. *Nucleic Acids Res.* 30:667–674.
29. Noordzij, J.G., N.S. Verkaik, M. Van Der Burg, L.R. Van Veelen, S. De Bruin-Versteeg, W. Wiegant, J.M. Vossen, C.M. Weemaes, R. De Groot, M.Z. Zdzienicka, et al. 2003. Radiosensitive SCID patients with Artemis gene mutations show a complete B-cell differentiation arrest at the pre-B-cell receptor checkpoint in bone marrow. *Blood*. 101:1446–1452.