RAG: A recombinase diversified

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Abstract

During B and T cell development, the lymphoid-specific proteins RAG1 and RAG2 act together to initiate antigen receptor gene assembly via a series of site-specific, somatic DNA rearrangements that are collectively termed V(D)J recombination. In the past 20 years, a great deal has been learned about the enzymatic activities of the RAG1-RAG2 complex. Recent studies have revealed several new and exciting regulatory functions of the RAG1-RAG2 complex. Here we discuss some of these functions and suggest that the RAG1-RAG2 complex nucleates a specialized subnuclear compartment that we term the V(D)J recombination factory.

To generate a diverse repertoire of antigen receptors, developing B cells and T cells undergo a complex series of DNA rearrangements collectively termed V(D)J recombination. This process is initiated by the lymphoid-specific proteins RAG1 and RAG2, which function together to generate site-specific DNA double-strand breaks that are then repaired via the classical non-homologous end-joining (NHEJ) pathway. While it is well established that RAG1-RAG2 can function as a recombinase, several recent studies have revealed that RAG1-RAG2 is actually a surprisingly multifaceted enzyme complex that plays an important role in ensuring that V(D)J recombination is faithfully executed and properly regulated in the cell. In this Review, we discuss the role of the RAG1-RAG2 complex in binding to accessible chromatin, mediating allelic pairing during V(D)J recombination, and channeling RAG-generated double-strand breaks towards the classical non-homologous end-joining (NHEJ) pathway (Fig. 1). We conclude by proposing a speculative model in which the RAG1-RAG2 recombinase functions within a specialized subnuclear compartment that we term the V(D)J recombination factory.

RAG1-RAG2 'reads' marks of accessible chromatin

Given the importance of generating functional B cell antigen receptors (BCRs) and T cell antigen receptors (TCRs) and the need to protect the integrity of the genome from inappropriate RAG-catalyzed double-strand breaks, it is not surprising that the process of V(D)J recombination is strictly regulated at multiple different levels. Specifically, V(D)J recombination exhibits tissue-specificity with recombination only occurring in lymphoid cells, lineage-specificity with complete immunoglobulin (Ig) gene rearrangement restricted to B cells and complete TCR gene rearrangement restricted to T cells, and developmental stage-specificity such that rearrangement only occurs in pro-B, pro-T, pre-B and pre-T cells. Additionally, V(D)J recombination is temporally ordered, such that D-J rearrangement precedes V-DJ rearrangement at the *Igh* and *Tcrb* loci, and cell cycle-regulated, with recombination restricted to the G0/G1 stage of the cell cycle. Finally, V(D)J recombination

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is subject to allelic exclusion, where productive rearrangement on one allele prevents rearrangement on the other allele, and isotypic exclusion of the light chain loci, by which productive rearrangement of *Igk* prevents rearrangement of *Igl* and vice versa. As the same RAG1-RAG2 recombinase catalyzes all V(D)J recombination events, and yet only a limited subset of all recombinationally competent gene segments are actually rearranged in a given cell at a given time, V(D)J recombination must be regulated by modulating the accessibility of the various antigen receptor loci to the RAG proteins. Although it is widely believed that alterations in chromatin structure are utilized to achieve this control, mechanisms linking specific aspects of chromatin structure to the function of the RAG recombinase have remained elusive. However, several new studies have provided insight into how chromatin structure may regulate V(D)J recombination.

Recently, an exciting new link between histone methylation and V(D)J recombination was identified. Using a variety of biochemical and biophysical assays, it was demonstrated that RAG2 contains a noncanonical plant homeodomain (PHD) finger that specifically recognizes histone H3 trimethylated at lysine 4 (H3K4me3) [1, 2]. Chromatin immunoprecipitation (ChIP) analysis revealed that H3K4me3 is enriched at gene segments poised to undergo V(D)J recombination[2, 3]. Interestingly, the pattern of H3K4me3 enrichment is consistent with the tissue-specificity, lineage-specificity and temporal ordering of antigen receptor gene rearrangement. For example, H3K4me3 is not present at the *Tcrb* or *Igh* loci in fibroblasts, and the *Igh* locus only exhibits H3K4me3 enrichment in pro-B cells while the *Tcrb* locus only exhibits H3K4me3 enrichment in pro-T cells. Moreover, the deposition of H3K4me3 and the initiation of recombination are essentially temporally coincident[3]. A high-resolution crystal structure of the RAG2 PHD finger bound to H3K4me3 revealed the molecular basis of H3K4me3-recognition and facilitated a functional analysis of the RAG2-H3K4me3 interaction (Fig. 1e)[2, 4]. Strikingly, mutations that abrogate RAG2 recognition of H3K4me3 severely impair V(D)J recombination *in vivo*[1, 2]. Similarly, reducing the abundance of H3K4me3 also leads to a marked decrease in V(D)J recombination[2]. Taken together, the data in these studies demonstrate that recognition of H3K4me3 by RAG2 is crucial for V(D)J recombination.

Although these studies revealed an intriguing new link between chromatin structure, the RAG recombinase, and V(D)J recombination, many questions remain. For example, given that H3K4me3 is found at many locations throughout the genome, how does the RAG1-RAG2 complex get specifically targeted to the antigen receptor loci? One potential answer is suggested by the crystal structure of the RAG2 PHD finger ($RAG2_{PHD}$) bound to H3K4me3[2, 4]. Comparing the crystal structures of the PHD finger of RAG2 to the PHD fingers of the transcription factors BPTF and ING2 bound to H3K4me3 suggested that whereas BPTF and ING2 can only recognize histone H3 lysine 4 trimethylation when the nearby arginine 2 residue is unmethylated (H3R2me0K4me3), RAG2 might be able to recognize histone H3 lysine 4 trimethylation when arginine 2 is also methylated. Consistent with this prediction, $RAG2_{PHD}$ not only recognizes histone H3 when it is symmetrically dimethylated at arginine 2 and trimethylated at lysine 4 (H3R2me2sK4me3)[4], but it actually binds this dually-modified form of histone H3 more tightly than it binds to H3K4me3[5]. Furthermore, the ability of ING2 to bind H3K4me3 was dramatically impaired by methylation of arginine 2[4]. Therefore, if poised antigen receptor loci are marked by H3R2me2sK4me3 while other portions of the genome are only marked by H3K4me3, then H3R2me2s might serve as a specificity factor that helps recruit the RAG1-RAG2 complex to the antigen receptor loci, and exclude other H3K4me3-binding complexes from these sites. Future experiments will elucidate the functional role of H3R2me2s in V(D)J recombination.

How does RAG2 recognition of H3K4me3 promote V(D)J recombination? There seem to be three general possibilities. First, the RAG2-H3K4me3 interaction may be required for

recruiting and/or retaining the RAG1-RAG2 complex at poised antigen receptor loci. This recruitment and/or retention function could be important prior to initial RSS binding and/or during synapsis of a 12/23 RSS pair. Second, the RAG2-H3K4me3 interaction may induce a conformational change in the RAG1-RAG2 complex, thereby allosterically activating the recombinase and stimulating chromosomal V(D)J cleavage. Such activation could occur via a simple conformational change or it could occur via displacement of an inhibitory group. Intriguingly, it has been shown that unlike the PHD fingers of BPTF and ING2, which have closed H3K4me3-binding "cages," RAG2 has an H3K4me3-binding channel that is open at the top. Furthermore, in the absence of an H3K4me3 peptide, this open channel can be filled from above with another peptide that positions a proline into the aromatic pocket that normally interacts with the trimethylated lysine 4 residue[4]. This finding suggests the potential for auto-regulation of RAG2 function. Perhaps, in the absence of H3K4me3, a disordered loop of RAG2 folds back upon itself in such a way that the H3K4me3-binding groove is filled from above by a self-peptide. With RAG2 in this conformation, the RAG1-RAG2 complex might exhibit suboptimal catalytic properties. However, in the presence of H3K4me3 (or H3R2me2s/K4me3), the methylated H3 tail would fill the H3K4me3-binding groove from below, displacing the self-peptide, and increasing the rate of V(D)J cleavage. Third, the RAG2-H3K4me3 interaction might stabilize the RAG1-RAG2 post-cleavage complex, helping the recombinase to bind cleaved signal and coding ends, thereby facilitating efficient 'hand-off' of V(D)J recombination intermediates to the NHEJ machinery. A great deal of further work will be required to distinguish between these possibilities. Undoubtedly, understanding how the interaction between RAG2 and H3K4me3 regulates chromosomal V(D)J recombination will provide important insights into both the mechanism of V(D)J recombination and the broader question of how chromatin-binding modules affect the function of chromosomal molecular machines.

The dependence of V(D)J recombination on H3K4me3 also raises the question of where the modified nucleosome is positioned with respect to the recombination signal sequence. Does the RSS have to be incorporated into a nucleosome bearing this modification or could it be in close proximity to or even at a considerable linear distance from this modified nucleosome? Although some groups were unable to detect V(D)J cleavage of an RSS within a mononucleosome[6, 7], others showed that RAG1-RAG2 can cleave nucleosomal substrates[8–10]; the latter findings suggest that it is possible for the RSS to be within the modified nucleosome and still participate in V(D)J recombination. On the other hand, the RAG2 PHD finger is separated from the RAG2 core by a flexible hinge which might allow RAG2 to bind H3K4me3 via its PHD finger while the RAG1-RAG2 complex contacts the RSS present in the linker DNA. Of course, depending on what role H3K4me3 plays in the reaction, the time at which H3K4me3 binding is important might be temporally distinct from the actual catalytic steps of the reaction. Future experiments should help to distinguish amongst these possibilities.

The functional importance of the RAG2- H3K4me3 interaction for V(D)J recombination also provides a new insight into the potential role of germline transcription, which is observed at antigen receptor loci and appears to precede V(D)J recombination at these loci (Fig. 1b). This so-called "sterile" transcription has long been known to occur, but its functional importance and relationship to V(D)J recombination has remained unclear. The principal mechanism for trimethylating H3K4 requires transcription, with the MLL-family of methyltransferases being recruited near promoters via recognition of the form of RNA polymerase II whose C-terminal domain is phosphorylated at serine 5. Therefore, the primary function for germline transcription might be to mark these loci with H3K4me3 to allow for efficient V(D)J recombination. If this is the case, an additional question is raised: how is H3K4me3 deposited across a large region of the locus, as opposed to being restricted to the promoter region of the few known germline transcripts that appear prior to V(D)J

recombination? At the murine *Igh* locus, for example, H3K4me3 is present across the 80 kb DJ domain[2], even though there is only one well-characterized, active promoter (DQ52) in this region prior to D_H-J_H recombination[11]. This discrepancy suggests that there are several, previously unidentified, promoters interspersed throughout the DJ domain (e.g. the poorly-defined promoters driving antisense transcription within this region[12, 13]) or that there is an additional mechanism that allows H3K4me3 to be deposited at a greater distance from the DQ52 promoter than has been observed for H3K4me3 deposition at other genes. This function of germline transcription in V(D)J recombination remains to be explored.

Finally, this study expands the range of regulatory functions that can be ascribed to the C-terminal, "non-core" portion of RAG2 (aa 384–527). In addition to recognizing methylated histone H3, the C-terminal portion of RAG2 also binds to phosphoinositides[14], suppresses RAG transposition[15–18], restricts V(D)J recombination to the G0/G1 stage of the cell cycle[19], and regulates V_H-DJ_H recombination[20–22]. We note that RAG2 PHD finger point mutants that are specifically deficient in H3K4me3-binding are more impaired in D_H-J_H recombination than core RAG2 (aa 1–383), which lacks the entire C-terminal regulatory region[2]. This apparent paradox may, in part, reflect the fact that core RAG2 lacks the G1/S degradation signal[23], and therefore accumulates to much higher quantities in developing B cells than the RAG2 PHD finger mutants[2]. Thus, if the RAG2-H3K4me3 interaction plays a role in targeting the RAG1-RAG2 recombinase to D_H and J_H gene segments, core RAG2 might be able to partially overcome its inefficient targeting by simple mass action. Additionally, if RAG2 is subject to PHD finger-dependent auto-inhibition as discussed above, then core RAG2 would be more efficient at V(D)J recombination than PHD finger mutants that are constitutively auto-inhibited due to their inability to bind H3K4me3. This phenomenon, wherein the presence of an impaired regulatory domain is more deleterious than its absence, was also seen in studies of nuclear hormone receptors[24] and Src kinase[25]. In any case, it seems clear that the C-terminal portion of RAG2 is an important regulatory region that affects V(D)J recombination in a variety of interesting ways.

RAG1-RAG2 mediates allelic pairing

While it is clear that localized molecular events such as histone methylation play an important role in regulating V(D)J recombination, it is becoming increasingly apparent that V(D)J recombination is also regulated by large-scale chromosome dynamics. In pro-B cells, which are poised to undergo V(D)J recombination, the *Igh* loci are repositioned from the peripheral nuclear lamina to a central region of the nucleus (Fig. 1a)[26], where these loci then undergo large-scale contraction[26] via a DNA looping mechanism(Fig. 1d)[27, 28]. A similar process of locus contraction also occurs at the *Tcra* and *Tcrb* loci in developing thymocytes[29]. *Igh* locus contraction is dependent upon the interleukin 7 receptor[26] and the transcription factors Pax5[30], YY1[31], and Ikaros[32], and may play a role in regulating the accessibility of distal V_H gene segments during V_H-DJ_H recombination. Although several factors have been implicated in *Igh* locus contraction, little is known about the initial process of subnuclear repositioning of the *Igh* locus. Nonetheless, it seems clear that large-scale chromosome dynamics play an important role in regulating antigen receptor loci accessibility during lymphoid development.

A very recent study extends this theme, demonstrating that during B cell development, homologous immunoglobulin alleles pair up in a lineage-specific and developmental stage-specific manner that correlates with the recombinational activity of the immunoglobulin loci (Fig. 1c)[33]. That is, in pre-pro-B and pro-B cells, which are undergoing D_H-J_H and V_H-DJ_H recombination respectively, the two *Igh* alleles pair with each other, while in pre-B and immature B cells, which are undergoing V -J recombination, the two *Igk* alleles pair with each other. Surprisingly, this allelic pairing seems to be dependent upon RAG1 expression,

but independent of the RAG1-RAG2-catalyzed V(D)J cleavage because it still occurs with a catalytically inactive form of RAG1. Thus, the RAG1-RAG2 complex seems to play a role in mediating allelic pairing during B cell development.

Of note, the role of RAG2 in this pairing, or more specifically, the histone-binding[2] and phosphoinositide-binding[14] activities of the RAG2 PHD finger, have not yet been explored. The question of how the RAG1-RAG2 complex influences allelic pairing also remains. Does it directly bind and synapse the homologous Ig alleles? Or does it play an indirect role, perhaps by serving as a molecular scaffold that interacts with other factors once these factors have bound to the Ig loci? Complementing *Rag1*$^{-/-}$ cells with binding-deficient RAG1 mutants[34–36] could help to distinguish between these possibilities. Also, what other factors are involved in the allelic pairing process? And finally, if the purpose of allelic pairing is to allow the two alleles to communicate with each other, thereby ensuring that productive recombination only occurs on one of the two immunoglobulin alleles, how is information transferred from one allele to the other? That is, how does allelic pairing enable one allele to assess the recombinational status of the other allele? Although many questions remain, the finding that the RAG1-RAG2 complex plays a role in mediating allelic pairing of immunoglobulin loci during B cell development is very exciting and certainly expands our view of how the RAG1-RAG2 complex helps to regulate V(D)J recombination.

RAG1-RAG2 promotes classical non-homologous end-joining

During lymphoid development, the RAG1-RAG2 complex initiates V(D)J recombination by binding to a pair of recombination signal sequences (RSS) and generating DNA double-strand breaks at the RSS-coding joint junctions. These double-strand breaks are then repaired via non-homologous end-joining to form a precise signal joint and an imprecise coding joint. It is well-established that the classical NHEJ (cNHEJ) machinery is required to properly repair the programmed double-strand breaks generated during V(D)J recombination. It has also been shown that mutations in RAG1[35, 37, 38] and RAG2[39] can impair the repair of signal ends and coding ends. However, it has been rather unclear why RAG-generated double-strand breaks are repaired by the classical NHEJ pathway as opposed to the alternative NHEJ (aNHEJ) pathway, which relies on microhomology between the broken ends that are being repaired.

A recent report suggests that RAG2 may play an important role in channeling V(D)J recombination intermediates towards the classical NHEJ repair pathway (Fig. 1f)[40]. Using a cellular V(D)J recombination assay that distinguishes between classical non-homologous end-joining and microhomology-driven alternative non-homologous end-joining, it was demonstrated that a RAG2 frameshift mutant (FS361) that is missing 22 residues more than are absent from RAG2$_{core}$ exhibits a greatly increased frequency of alternative NHEJ-mediated repair of signal ends and coding ends. This mutant allows V(D)J recombination intermediates to access the alternative NHEJ pathway in wild-type cells, where the classical NHEJ machinery is intact, and it also rescues the end-joining defects found in cNHEJ-deficient cells. The fact that this mutation allows broken ends to be repaired by the alternative NHEJ pathway, even when the classical NHEJ machinery is fully functional, suggests that the C-terminal portion of RAG2 plays a key role in channeling RAG-generated DNA double-strand breaks to the classical NHEJ pathway. Moreover, the observation that the FS361 mutant exhibits significantly more aNHEJ-mediated repair of signal ends and coding ends than does RAG2$_{core}$ suggests that residues 362–383 of RAG2 may play an especially important role in directing RAG-generated double-strand breaks to the classical NHEJ pathway.

How does the C-terminal portion of RAG2 determine whether V(D)J recombination intermediates are repaired via classical or alternative NHEJ? Perhaps the C-terminal portion of RAG2 plays a role in stabilizing the RAG1-RAG2 post-cleavage complex, as has been demonstrated for other joining-deficient RAG mutants[37, 41]. Alternatively, as it has recently been found that the N-terminal portion of RAG1 forms protein-protein interactions (either directly or indirectly) with two components of the cNHEJ machinery (Ku70 and Ku80)[42], perhaps the C-terminal portion of RAG2 also interacts with components of the cNHEJ machinery. Such an interaction could promote the efficient transfer of signal ends and coding ends from the RAG1-RAG2 post-cleavage complex to the cNHEJ pathway. Future studies will determine the mechanism(s) by which the C-terminal portion of RAG2 influences the fate of RAG-generated double-strand breaks.

Model

Given the myriad functions required to effect the complex chromosomal gymnastics that occur during antigen receptor gene rearrangement, including subnuclear repositioning[26], allelic pairing and unpairing[33], reversible locus contraction[26, 29], dynamic chromosomal looping[27, 28], and long-range 12/23 synapsis and double-strand break repair, one might wonder how these various functions are integrated within the nucleus to ensure the faithful execution of V(D)J recombination. We hypothesize that antigen receptor gene assembly may occur within a specialized subnuclear compartment that we will term the V(D)J recombination factory (Fig. 2). This specialized compartment, which likely exists within a central, euchromatic region of the nucleus[26], would localize the factors required to alter the large-scale chromosomal architecture of the antigen receptor loci (e.g. Pax5[30], YY1[31], Ikaros[32], ATM[43], and potentially many others), the factors required to alter the local chromatin structure of the antigen receptor loci (e.g. ATP-dependent chromatin remodeling enzymes[9, 10, 44, 45], H3/H4 lysine-acetyltransferases (KATs)[9, 45–48], and H3K4 lysine-methyltransferases (KMTs)[1, 2]), as well as the RAG1-RAG2 recombinase and the cNHEJ machinery. We hypothesize that the V(D)J recombination factory exists as a single subnuclear compartment that is uniquely equipped to carry out the process of chromosomal V(D)J recombination. Only a single compartment would be required since typically, only a single antigen receptor locus is undergoing V(D)J recombination at any given point in time. Such a unique site would be in contrast to transcription factories, which seem to exist at multiple sites within the nucleus likely reflecting that, at any point in time, many different genes are being transcribed from several different chromosomes. In light of the many ways that RAG1-RAG2 complex seems to influence other processes involved in V(D)J recombination including allelic pairing and unpairing and choice of double-strand break repair pathways, and given that the RAG1-RAG2 recombinase is the central protein machine driving V(D)J recombination, we suggest that the RAG1-RAG2 complex is the organizing factor that nucleates the V(D)J recombination factory. Other factors would then be recruited to and retained within this subnuclear compartment. By physically bringing together all of the factors that influence chromosomal V(D)J recombination, and concentrating them within a V(D)J recombination factory, the cell could functionally couple the numerous processes that occur during V(D)J recombination, thereby enhancing both the efficiency and the regulation of antigen receptor gene rearrangement. If the cell excludes non-antigen receptor loci from this subnuclear compartment, then the V(D)J recombination factory might also help to minimize the formation of spurious RAG-generated double-strand breaks at cryptic RSSs[49, 50] and non-B-form DNA[51] throughout the genome, as well as decrease the opportunity for deleterious interchromosomal translocations[52–55].

Conclusion

This is an exciting time in the field of V(D)J recombination. Recent studies have demonstrated that the RAG1-RAG2 complex is a talented and versatile molecular machine that does much more than simply catalyze the phosphoryl transfer reactions required to generate DNA double-strand breaks. In addition to cutting DNA, the RAG1-RAG2 complex also interacts with H3K4me3, plays a role in immunoglobulin allelic pairing, and helps determine whether the broken ends generated during V(D)J recombination are repaired via classical NHEJ, alternative NHEJ, or homologous recombination. A great deal of future research will be required to unravel the mechanisms underlying these processes and to test the hypothesis that antigen receptor gene assembly occurs within specialized V(D)J recombination factories. Nonetheless, it seems clear that in addition to its central role in the development of the vertebrate adaptive immune system, V(D)J recombination is also becoming a valuable system to study many fundamental questions pertaining to chromatin accessibility, nuclear dynamics, long-range chromosomal interactions, and DNA double-strand break repair.

Acknowledgments

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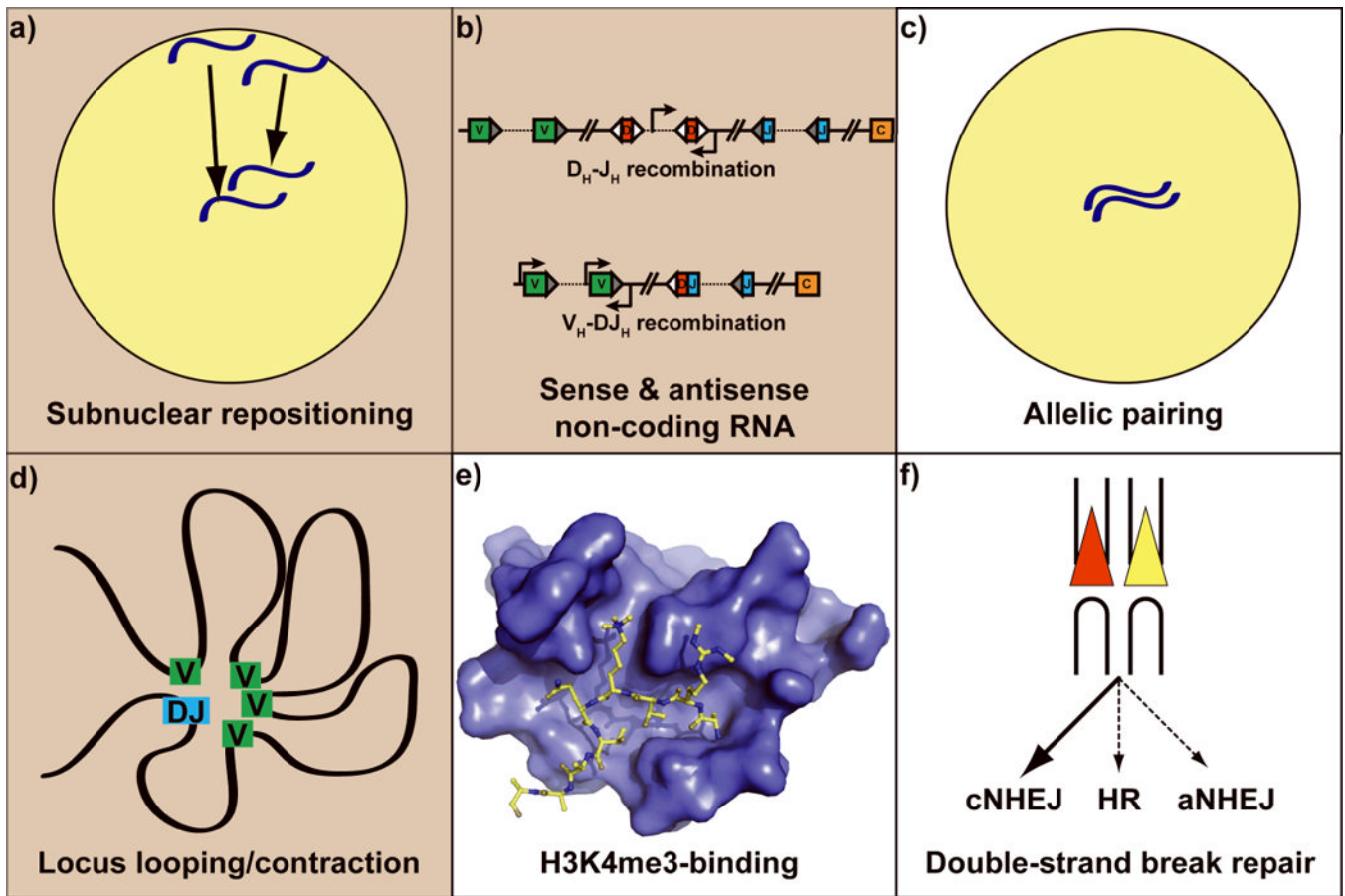
Figure 1. Multilayered regulation of V(D)J recombination
Regulatory mechanisms that are RAG-dependent are shown in white panels (**c**, **e**, and **f**), and those that are RAG-independent are in tan (**a**, **b**, and **d**). **a)** Subnuclear localization of antigen receptor loci. Within the nucleus (yellow circle) the homologous *Igh* alleles are depicted in blue. Arrows indicate the centripetal movement of *Igh* alleles prior to initiation of V(D)J recombination at this locus; **b)** Sense and antisense non-coding RNA. V_H (green), D_H (red), J_H (blue), and C_μ (orange) gene segments are shown. Right- and left-facing arrows represent sense and antisense transcription start sites, respectively; **c)** Allelic pairing. As in **a**, the nucleus is shown as a yellow circle and homologous *Igh* alleles are depicted in blue; **d)** Locus looping and contraction. The curved black line is the *Igh* locus, with V_H gene segments in green and rearranged DJ_H gene segments in blue. As a result of chromosomal looping, the distance between V_H gene segments and the DJ_H gene segment is decreased; **e)** Recognition of histone H3 methylation. A space-filling model of the RAG2 PHD finger is shown in blue, bound to the N-terminal tail of histone H3 (shown in yellow), which is trimethylated at lysine 4 and symmetrically dimethylated at arginine 2; **f)** Preferential usage of the classical NHEJ (cNHEJ) double-strand break repair pathway. The RAG recombinase aids in directing V(D)J recombination intermediates to cNHEJ and away from homologous recombination (HR) and alternative NHEJ (aNHEJ). Signal ends: red and yellow triangles; coding ends: U-shaped black lines.
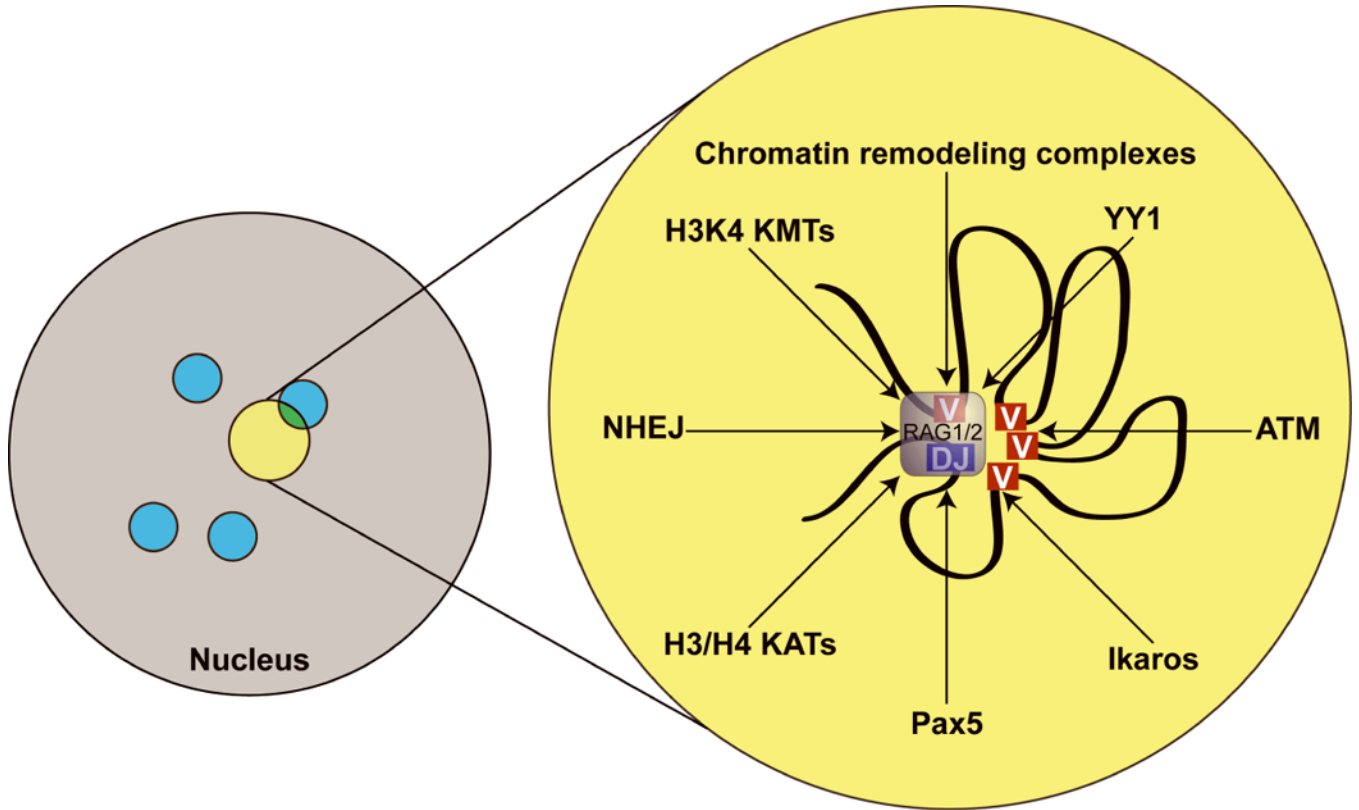
Figure 2. Model depicting a putative V(D)J recombination factory
Within the nucleus (gray circle), the V(D)J recombination machinery may exist as a
specialized, centrally-located, subnuclear compartment (yellow circle). This compartment is
likely to be distinct from transcription factories (blue). An expanded view of the V(D)J
recombination factory is shown on the right. *Igh* locus: curved black line; V_H gene
segments: red, a rearranged DJ_H gene segment: blue. The RAG1-RAG2 recombinase (light
purple) is shown synapsing the DJ_H gene segment and one of the V_H gene segments just
prior to V_H-DJ_H recombination. Factors that are likely to be enriched within the V(D)J
recombination factory are indicated. The central position of the RAG recombinase within
this figure reflects the hypothesis that the RAG1-RAG2 complex nucleates the factory,
while arrows pointing towards the RAG1-RAG2 complex represent the recruitment of
additional regulatory factors by the RAG1-RAG2 complex.