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The Role of Mechanistic Factors in Promoting Chromosomal Translocations Found in Lymphoid and Other Cancers

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Abstract

Recurrent chromosomal abnormalities, especially chromosomal translocations, are strongly associated with certain subtypes of leukemia, lymphoma and solid tumors. The appearance of particular translocations or associated genomic alterations can be important indicators of disease prognosis, and in some cases, certain translocations may indicate appropriate therapy protocols. To date, most of our knowledge about chromosomal translocations has derived from characterization of the highly selected recurrent translocations found in certain cancers. Until recently, mechanisms that promote or suppress chromosomal translocations, in particular, those responsible for their initiation, have not been addressed. For translocations to occur, two distinct chromosomal loci must be broken, brought together (synapsed) and joined. Here, we discuss recent findings on processes and pathways that influence the initiation of chromosomal translocations, including the generation fo DNA double strand breaks (DSBs) by general factors or in the context of the Lymphocyte-specific V(D)J and IgH class-switch recombination processes. We also discuss the role of spatial proximity of DSBs in the interphase nucleus with respect to how DSBs on different chromosomes are justaposed for joining. In addition, we discuss the DNA DSB response and its role in recognizing and tethering chromosomal DSBs to prevent translocations, as well as potential roles of the classical and alternative DSB end-joining pathways in suppressing or promoting translocations. Finally, we discuss the potential roles of long range regulatory elements, such as the 3'IgH enhancer complex, in promoting the expression of certain translocations that are frequent in lymphomas and, thereby, contributing to their frequent appearance in tumors.

1. CHROMOSOMAL TRANSLOCATIONS IN CANCER

1.1. Overview

In 1914, Theodor Boveri postulated the somatic mutation theory of cancer (Boveri, 1914). Microscopic analysis of chromosomal segregation in dividing cells led him to hypothesize that changes in the chromosome constitution of a single cell are the driving force of tumorigenesis. Boveri suggested that a cancer originates from a single cell that has acquired an abnormal chromosomal constitution, and that this abnormality, when passed on to all the descendants overcomes a normal inhibitory mechanism, thereby causing rapid cell proliferation. Today, it is widely accepted that cancer is a genetic disease, caused by genomic aberrations, including point mutations or chromosomal translocations or

amplifications that lead to activation of oncogenes or mutations or chromosomal deletions that inactivate tumor suppressor genes (reviewed in Fröhling and Döhner, 2008; Stratton *et al.*, 2009).

Chromosomal translocations are abnormalities caused by visibly aberrant rearrangements of large pieces of chromosomal DNA. Translocations are one of several subtypes of chromosomal rearrangements traditionally distinguished as deletions, amplifications, inversions, duplication/insertions, and translocations. In this context, there are two general types of translocations. Reciprocal translocations appear as a simple swap between two nonhomologous chromosome arms such that the chimeric products are monocentric in metaphase. Nonreciprocal translocations on the other hand can lead to large deletions or duplications of chromosomal segments. Nonreciprocal translocations may or may not originate mechanistically by the same general mechanisms as reciprocal translocations. In either case, the pattern with which the nonhomologous chromosomes swap yield chimeric chromosomes that generate an imbalance; for example, dicentric chromosomes that can lead to deletions or amplifications as the cells progress through the cell cycle. Thus, the definition of reciprocal versus nonreciprocal translocation refers to the outcome of a rearrangement as visualized in cytogenetic analysis and not necessarily to the underlying mechanistic distinctions. The commonality between the initiating molecular mechanistic paths by which visible karyotypic changes from a normal cell are introduced into a premalignant clone, whether reciprocal or nonreciprocal translocations, deletions, amplifications, inversions, and insertions is not well defined at present (Zhang *et al.*, 2009). Further resolution of the mechanistic factors that lead to these two general types of translocations in mammalian cells, for example, when translocation-initiating DNA double-strand breaks (DSBs) are introduced during the cell cycle and when during the cycle and how they are repaired (e.g., Zhu *et al.*, 2002), awaits the development of new techniques to follow the initial generation of translocations before they are further molded by cellular and oncogenic selection.

1.2. Oncogenic translocations in cancer frequently have DSB intermediates

Chromosomal translocations are fundamental pathogenetic events in cancer, both with respect to tumor onset and tumor progression. Recurrent oncogenic translocations are common features of hematopoietic malignancies such as leukemia and lymphomas (Küppers and Dalla-Favera, 2001). Likewise, genomic instability in the form of translocations and related deletions and amplifications occurs in the context of solid tumor progression. Solid tumors, such as certain brain tumors, as well as prostate and lung cancers, also can contain recurrent translocations (Mitelman *et al.*, 2007). Indeed, a major goal of the large cancer genome project is to better define genomic alterations in tumor cells, including translocations, deletions, inversions, and amplifications. DSBs are common intermediates in such genomic aberrations. DSBs can be generated by normal metabolic processes, by genotoxic agents including agents commonly used to treat cancer, and by the programmed processes of V(D)J recombination and immunoglobulin (Ig) heavy (H) chain (IgH) class switch recombination (CSR) in T and/or B lymphocytes. When DSBs occur, highly conserved DNA repair pathways efficiently rejoin broken ends to preserve the genome integrity. Nonetheless, repair sometimes fails, and the resulting unresolved DSBs can lead to chromosomal translocations and other genomic aberrations.

1.3. Chromosomal translocations in hematological malignancies

Chromosomal translocations are most well characterized in hematological malignancies. Several online databases are maintained with frequent updates where all chromosomal abnormalities in the cancer genetic literature are tracked in a searchable form (http://cgap.nci.nih.gov/Chromosomes/Mitelman, http://atlasgeneticsoncology.org/,

http://www.sanger.ac.uk/genetics/CGP/Census/; Futreal *et al.*, 2004). At present, more than 300 recurrent chromosomal translocations have been annotated. While some translocations may be late events that occur during the progression of a tumor, there is now clear evidence translocations associated with certain cancers actually contribute to transformation at an early stage of tumorigenesis (Stratton *et al.*, 2009). Hematological malignancies, which account for about 10% of human cancers (Küppers and Dalla-Favera, 2001), frequently harbor characteristic recurrent chromosomal translocations that can activate oncogenes by several different mechanisms. One mechanism is the generation of chimeric proteins that result from chromosomal translocations that fuse the coding sequences of two different genes lying on the two translocation partner chromosomes. Another mechanism is the linkage of a potential oncogene to a regulatory element in a different locus; for example, the enhancer elements found in Ig loci, which lead to the misregulated expression of a translocated, but otherwise intact, structural gene. Indeed, more than 50% of leukemias and lymphomas carry reciprocal chromosomal translocations that recur among different patients and that are highly associated with particular cancer subtypes (Zhang and Rowley, 2006). Examples of recurrent translocations that lead to either novel fusion proteins or deregulated oncogene expression are discussed below.

A translocation called the Philadelphia chromosome by cytogeneticists was the first example of a consistent aberrant chromosomal translocation in a human cancer (Nowell and Hungerford, 1960). The Philadelphia chromosome was initially visualized as an unusually short chromosome that was recurrent in chronic myelogenous leukemia (CML) cells, and subsequently characterized in more detail as a t(9;22) (q34;q11) translocation (Rowley, 1973). In the 1980s, the crossover site on the Philadelphia chromosome was cloned and found to encode a composite gene in which all but the more 5′-exons of the cellular ABL gene (a cellular homologue of the v-Abl Abelson murine leukemia virus oncogene) on chromosome 9 had become joined to a gene termed BCR (for breakpoint cluster region; Rowley, 1973) on chromosome 22. The BCR–ABL fusion protein retained the tyrosine kinase activity of the parent ABL gene and was shown to be oncogenic (Bartram *et al.*, 1983; de Klein *et al.*, 1982; Groffen *et al.*, 1984; Heisterkamp *et al.*, 1983). The BCR-encoded portion of the protein was found to mediate permanent oligomerization of the chimeric protein, which enforced a constitutive and altered kinase activity (Goldman and Melo, 2003).

Having pinpointed the misregulated tyrosine kinase as a driving force in CML, a targeted approach in which tyrosine kinase inhibitors were screened for efficacy and specificity led to the discovery of imatinib mesylate (Gleevec), marking a stunning advance in the treatment of CML and other diseases exhibiting the Philadelphia chromosome (Druker *et al.*, 2006). Oncogenic translocations, as illustrated by the Philadelphia chromosome and Gleevec example, can represent an Achilles' heel of a tumor, revealing effective targets for drug therapy. Where the unregulated proliferation in cancer cells is largely due to abnormal oncogene expression via formation of a fusion protein or deregulated transcription, it is possible that inactivation of a single oncogene can result in the elimination of most, if not all, tumor cells (Felsher, 2008).

The other classic mechanism by which a reciprocal chromosomal exchange can be tumorigenic is by leading to changes in the level and/or specificity of the expression of a potential cellular oncogene. There are many examples of this form of deregulated protein expression, particular in B and T lymphoid malignancies in which potential oncogenes are placed under the control of tissue-specific gene regulatory elements that lie, respectively, within Ig or T cell receptor (TCR) loci. The classical example of a recurrent translocation that leads to such deregulated proto-oncogene expression is the t(8;14)-related translocations observed in Burkitt's lymphoma (Janz, 2006). In the context of this translocation, the *c-Myc*

(MYC1) gene, normally on chromosome 8 is fused to an *Ig* locus, most commonly the *IgH* locus near the telomere of the long arm of chromosome 14, but also to the *Ig*κ or *Ig*λ light chain loci on the long arm of chromosome 2 or long arm of chromosome 22, respectively (Janz, 2006). The *c-Myc* proto-oncogene was first identified as the human homolog of the oncogene found in an avian myelocytomatosis retrovirus and it encodes the C-MYC transcription factor. Dramatically increased expression of C-MYC, promotes transition of the cell cycle from G1 to S phase, which favors tumor-associated clonal bursts and further tumorigenesis (Küppers, 2005).

Other classes of human tumors similarly have translocations that deregulate expression of oncogenes such as *Bcl-1* or *Bcl-6* by fusing them with *IgH* or *IgL* loci (Küppers and Dalla-Favera, 2001). Such translocations deregulate *c-Myc* (or other cellular oncogenes) by putting them under the control of Ig regulatory elements. Both B cell lymphomas and plasmacytomas in mouse tumor models also can have similar recurrent translocations between *IgH or IgL* loci and the *c-Myc* locus, which deregulate *c-Myc* expression (Janz, 2006). While it was originally thought that the intronic enhancer within the *IgH* locus was a key element in mediating such deregulated expression in translocations involving *IgH*, recent studies of mouse B cell lymphoma models have shown that the *IgH* 3′-regulatory region (IgH3′RR), downstream of the *IgH* locus, plays a key role through its ability to activate oncogenes over long distances (Gostissa *et al*., 2009a; see Section 3).

Amplification of the *c-Myc* gene have also been found in association with V(D)J recombination-initiated, nonreciprocal translocations to the *IgH* locus (referred to a complex translocations with amplifications or “complicons”) in mouse progenitor B cell lymphomas (Difilippantonio *et al.*, 2002; Zhu *et al.*, 2002) and have been occasionally been observed in human B cell lymphomas and multiple myelomas (Janz, 2006; Martín-Subero *et al.*, 2005). In addition, recent studies have implicated recurrent gene amplifications in the context of complicons initiated by V(D)J recombination-associated DSBs in the *TCR*δ locus in mouse thymic lymphomas that arise in the context of deficiency for the ataxia telangiectasia mutated (ATM) gene (Zha *et al.*, 2010).

1.4. Chromosomal translocations in epithelial tumors

Recurrent chromosomal translocations, excluding amplifications, have not been as frequently described in the context of epithelial carcinomas as in hematological malignancies. This might in some part reflect technical limitations in the generation of metaphase chromosome spreads from epithelial tumors. Moreover, another significant feature of epithelial neoplasms is that they often evolve complex karyotypes during tumor progression so that the multiple cytogenetic changes become difficult to interpret, and clonal markers are not identifiable (Mitelman *et al.*, 2007). Nonetheless there are examples of carcinomas in which some fraction of cases exhibit characteristic gene fusions brought about by recurrent translocations (reviewed in Brenner and Chinnaiyan, 2009).

About ~50% of papillary thyroid carcinomas (PTC) have translocations which connect the RET gene on chromosome 10 to a 5′ fusion partner that both mislocalizes and activates it. The specific identity of the protein can vary; the basic requirement appears to be that it confers dimerization capability (Nikiforov, 2008; Zitzelsberger *et al.*, 2010). Likewise, about 50% of prostate cancers overexpress ETS transcription factors due to a translocation that puts genes such as ERG or ETV1, ETV4 or ETV5 under the control of the 5′-untranslated region of an androgen responsive gene, TMPRSS2. This mechanism of oncogene activation via translocation is reminiscent of the mechanism of misregulated expression of *c-Myc* or other oncogenes when translocated to an Ig locus in lymphoid tumors. However, while the TMPRSS2–ETS family fusions are common in premalignant prostate lesions, they are insufficient for the initiation of tumors in mouse models (Carver *et*

al., 2009a). More recent work suggests that overexpressed ERG must cooperate with other mutations that inactivate a tumor repressor in promoting the progression from neoplasia to carcinoma (Brenner and Chinnaiyan, 2009; Carver *et al.*, 2009b).

Testing NIH 3T3 cells with a retrovirally delivered cDNA library prepared from a lung cancer specimen led to the description of an inversion within the long arm of chromosome 2 that joins the ALK (anaplastic lymphoma kinase) and EML4 (echinoderm microtubule-associated protein-like 4) loci (Soda *et al.*, 2007). The EML4–ALK fusion protein was shown to have tyrosine kinase activity and the transcript was detected in a significant fraction of patients. However, since then, the detection of EML4–ALK protein in non-small-cell lung cancers (NSCLCs) exhibiting fusion transcripts has been variable and the possibility that this particular rearrangement defines a therapeutic target remains to be determined (Martelli *et al.*, 2009; Soda *et al.*, 2008). An independent phosphotyrosin proteomics-based approach also detected ROS kinase (highly expressed in NSCLCs) fused to the transmembrane portions of either the SLC34A2 or CD74 genes in lung tumors (Rikova *et al.*, 2007).

1.5. Chromosomal translocations and cancer development

1.5.1. Roles of translocations in cancer—It has become clear that some translocations involving certain genomic loci are highly associated with specific tumor types (Mitelman *et al.*, 2007). In cases where the approach of targeting the chimeric protein produced by chromosomal translocation leads to a successful therapy (Hughes *et al.*, 2006), there is little room for doubt that tumorigenesis depends upon the acquisition and expression of the translocation product. In less certain situations, *in vivo* and *in vitro* methods have been used to investigate the role of particular translocations. For example, expression of the 210-kDa BCR–ABL protein (the product of the Philadelphia chromosome) in retro-virally transduced bone marrow cells is sufficient to cause a CML-like disease in mice (Pear *et al.*, 1998; Zhang and Ren, 1998). Likewise, transgenic overexpression of the *c-Myc* oncogene, deregulated by linkage to an *IgH* or *IgL* enhancer element at a level comparable to the deregulated expression resulting from Ig/Myc translocations, clearly demonstrated that the deregulated oncogene expression that results from such a chromosomal rearrangement can lead to the development of malignancy (reviewed in Morgenbesser and DePinho, 1994). However, even in the case of the MYC transgenic models, the tumors that arise are clonal, indicating that additional genetic alterations are involved.

The BCL-2 protein has antiapoptotic activities and thus can promote the survival of aberrant cells otherwise targeted for death via apoptosis (Youle and Strasser, 2008). The t(14;18) translocation, which fuses the *IgH* and *BCL-2 loci*, is a common genetic aberration found in certain human lymphoid malignancies. About 90% of follicular lymphomas and 20–30% of diffuse large B cell lymphomas have translocations that lead to deregulated expression of the translocated *bcl-2* gene (Küppers, 2005). Notably, over 50% of healthy individuals have small numbers of B cells with t(14;18) translocation that never progress to cancer (Schüler *et al.*, 2003). The reason for this is that even though high levels of the BCL-2 protein protect cells from early death by apoptosis, deregulation of BCL-2 alone is not sufficient for the development of B cell lymphomas. Thus, there is a requirement for additional genetic events. In this regard, mice carrying an *IgH–bcl-2* fusion transgene exhibited an abnormal expansion of resting B cells and lymphoid hyperplasia (McDonnell *et al.*, 1989). Yet, only after about 16 months, roughly 10% of these transgenic mice developed high-grade diffuse large B cell lymphomas. Such lymphomas that do develop in these older *IgH–bcl-2* transgenic mice frequently exhibit a rearranged *c-Myc* gene (McDonnell and Korsmeyer, 1991). Thus, studies with this mouse model provide a clear illustration that progression from benign follicular hyperplasia to malignant lymphoma requires multiple genetic changes.

1.5.2. Passenger versus driver translocations—Major efforts and substantial resources are now being devoted to projects aimed at characterizing cancer genomes, with the goal of better elucidating sets of mutations in cancer cells (Stratton *et al.*, 2009). However, such studies only examine the final outcome of the multistep transformation process, and the complex set of genomic alterations obtained can be difficult to fully interpret. Thus, while the cancer genome sequencing projects are data-rich, many of the genetic alterations in given tumors may not play a critical role in tumor development (Stratton *et al.*, 2009). The concept of "driver" versus "passenger" translocations usefully distinguishes between mutations that are causally implicated in the initiation of oncogenesis and those that may occur, even recurrently, without being integral to the process. Indeed, it now appears that the complexity of genomic alterations in cancer is much higher than expected. In particular, making the distinction between real oncogenic "driver" mutations or translocations from "passenger" events can be difficult.

Cancers, as illustrated by the *c-Myc* translocations in the lymphomas arising in *IgH–bcl-2* transgenic mice, may have required more than one driver to take hold, and likely the number of drivers will vary between cancer types. Moreover, mouse B cell lymphoma modeling studies have suggested that mechanistic factors, in the absence of selection, can drive the appearance of recurrent translocations in tumor progenitor cells at sufficiently high frequency that they appear as recurrent "passenger" translocations in the tumors (Wang *et al.*, 2009). In this context, some recurrent translocations, like those in advanced stage prostate cancer, could theoretically be promoted by mechanistic factors, such as proximity induced by androgen-dependent transcription in early stage tumors (Lin *et al.*, 2009; Mani *et al.*, 2009). Also, mechanistic factors have been implicated in promoting the appearance of the translocation of one oncogene versus another one with equivalent transforming activity in mouse tumor models (Gostissa *et al.*, 2009b; see below). In meeting the goal of identifying all driver mutations within cancer genomes, it will be necessary to more fully understand the mechanistic factors that can give rise to adventitious passenger translocations or promote particular types of recurrent translocations. The influence of mechanistic factors on translocations will be discussed in more depth below.

2. DNA RECOMBINATION IN B AND T CELL DEVELOPMENT

2.1. Overview

The V(D)J recombination events employed to assemble antigen receptor variable region exons in developing B and T lineage cells and the IgH CSR events that exchange *IgH* constant region exons in mature B cells are all initiated by DNA DSBs (Fig. 4.1). These lymphocyte-specific programmed DNA DSBs are normally repaired by general cellular DNA repair pathways that join broken DNA ends in the absence of large regions of homology and which are termed end-joining pathways. As will be outlined in more depth below, abnormalities in these lymphocyte-specific DNA breakage and joining pathways, whether it be at the introduction or joining of the programmed DSBs (or both), represent a major source of potential initiating events for chromosomal translocations that lead to lymphoid malignancies.

2.2. V(D) J recombination

Ig and TCR receptor variable region exons are assembled from V, (D), and J gene segments. Ig heavy chain and *TCR*β and *TCR*δ variable exons are assembled from V, D, and J segments; while *IgL* and *TCR*α and *TCR*γ variable region exons are assembled from just V and J segments (Dudley *et al.*, 2005). While the overall organization and content of individual Ig and TCR loci varies, each has numerous V, D, and J segments spread over large chromosomal distances. For example, the mouse *IgH* locus has 4 J segments, 11 D

segments, and hundreds of V segments organized as clusters that are spread over several megabases at the telomeric end of chromosome 12 (Fig. 4.1). The process of "V(D)J recombination" assembles the germ line V, D, and J segments into Ig and TCR variable region exons, respectively, in developing B and T lymphocytes. The *TCR*α and *TCR*δ locus have an unusual organization in that the *TCR*δ locus is fully contained within the *TCR*α locus, such that *TCR*α V(D)J recombination deletes the *TCR*δ locus (Dudley *et al.*, 2005). This unusual organization was the source of some confusion regarding the recurrent translocations that appear in ATM-deficient mouse thymic lymphomas. Thus, ATM-deficient thymic lymphomas were for years thought to have recurrent translocations that involved aberrant *TCR*α locus V(D)J recombination leading to translocations and oncogene activation by the *TCR*α enhancer; but, in fact, more recent studies have shown that these translocations arise during attempted *TCR*δ locus recombination and are independent of the *TCR*α enhancer (Zha *et al.*, 2010).

Recombination activating gene 1/2 (RAG) endonuclease initiates V(D)J recombination by cleaving V, D, and J segments (Oettinger *et al.*, 1990; Schatz and Baltimore, 1988; Schatz *et al.*, 1989), which are then joined by general cellular end-joining pathways to form V(D)J exons (Taccioli *et al.*, 1993). Thus, the "V(D)J recombinase" is comprised of both lymphoid-specific and generally expressed components. The RAG target site, called a recombination signal sequence (RSS), consists of seven base pair palindromic heptamer (canonical heptamer is CACAGTG) separated by a spacer of 12 or 23 base pairs from an AT-rich nonamer (Hesse *et al.*, 1989; Max *et al.*, 1979; Sakano *et al.*, 1979). The RAG proteins follow a "12/23" rule, in that two gene segments will be cleaved only if one segment is abutted by a 12-bp spacer RSS, and the other by an RSS with a 23-bp spacer (Tonegawa, 1983). The RSS motifs can vary in sequence, within limits, from one gene segment to the next and still support RAG cleavage. This is significant because there are many sequences in a mammalian genome that fortuitously resemble RSSs and which may be targeted for cleavage in developing B and T cells (Ferguson and Alt, 2001; Kim *et al.*, 2000), or even in more mature B cells (Wang *et al.*, 2009), that express RAG. As will be discussed later (Section 3), the V(D)J recombination process entails a certain degree of risk, and evidence of this risk is seen as oncogenic translocations in some lymphoid and myeloid tumors.

V(D)J recombination can be divided into several steps (Dudley *et al.*, 2005) (Fig. 4.2). Once an appropriate 12/23 pair of RSSs are engaged by RAG, one strand at the site just between each RSS and its adjacent gene segment is hydrolyzed. The 3′-OH at the break is then used as the nucleophile in the attack of the second strand just opposite. This process creates a pair of DSBs, at which each RSS has a blunt 3′-hydroxylated DSB end and each potential V, D, and J coding gene segment has a hairpin terminus. Notably, the RAG proteins carry out a transposition-related reaction and appear to have evolved from a primordial transposase; these potentially harmful transposition activities of the RAG proteins are normally suppressed by regions of the RAG proteins not required for normal catalytic activity (Mundy *et al.*, 2002; Sekiguchi *et al.*, 2001). In the second step of the V(D)J recombination reaction, the two broken RSS ends and two broken coding ends are, respectively, joined by the generally expressed classical nonhomologous DNA end-joining pathway (C-NHEJ; see below for more detailed description). The RSS ends are directly ligated without further processing; but the coding end hairpins must be opened and usually further processed before joining. All but one of the known proteins (Tdt) (Alt and Baltimore, 1982; Gilfillan *et al.*, 1993; Komori *et al.*, 1993) involved in trimming and joining the coding ends are general DNA repair factors in the C-NHEJ pathway (see below).

RAG expression is restricted primarily to developing B and T lymphocytes in the bone marrow and thymus, respectively (Monroe *et al.*, 1999; Yannoutsos *et al.*, 2001; Yu *et al.*,

1999); although some recent studies suggest that it may also occur in certain mature B lymphocytes (Wang *et al.*, 2009). RAG expression is also restricted to the G1 phase of the cell cycle (Desiderio *et al.*, 1996). Gene targeted mutations showed that RAG-1 and RAG-2 are necessary for initiation of V(D)J recombination *in vivo* (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992); thus, RAG-1 or -2 deficient mice are blocked in T and B cell differentiation and exhibit a severe combined immunodeficiency (SCID). In humans, hypomorphic, "leaky" mutations of the RAG genes are the most common cause of Omenn Syndrome (OS), a combined immunodeficiency associated with severe organ damage due to infiltrating activated, anergic, and oligoclonal T lymphocytes (Villa *et al.*, 1999).

The RAG endonuclease appears to have additional functions beyond RSS recognition and cleavage that might serve to direct its activity and, thus, impact on its potential to initiate translocations. A PHD finger in the C-terminal of RAG-2 has been identified recently by several groups (Liu *et al.*, 2007; Matthews *et al.*, 2007; Ramón-Maiques *et al.*, 2007). This motif specifically recognizes trimethylated histone H3 tail (H3K4me3), which leads to RAG-2 binding to sites of this histone modification genome wide and not just within *Ig* loci (Ji *et al.*, 2010). In addition, recent biochemical studies suggested H3K4me3 not only tethers the RAG enzyme complex to a region of DNA, but also induces a substantial increase in the catalytic turnover number (*k*(cat)) of the RAG complex (Shimazaki *et al.*, 2009). Of great relevance to potential roles in translocations, several studies have indicated that a postcleavage RAG1/2 complex is involved in later stages of V(D)J recombination and that RAG may be required to direct the repair of V(D)J recombination DSBs by general cellular DSB response and repair machinery (Huye *et al.*, 2002; Qiu *et al.*, 2001; Yarnell *et al.*, 2001). Most notably, the C-terminus of RAG-2 is important in ensuring that RAG-generated DSB intermediates are delivered to the C-NHEJ machinery (Corneo *et al.*, 2007; Cui and Meek, 2007; Lee *et al.*, 2004). If the RAG-2 protein contains a frameshift replaces the 166 carboxy-terminal amino acids with 27 novel amino acids, it forms a mutant RAG complex that will be able to catalyze V(D)J recombination in NHEJ-deficient cells via an alternative end-joining (A-EJ) pathway (Corneo *et al.*, 2007). Notably, A-EJ appears potentially more prone to catalyzing translocations than C-NHEJ (Simsek and Jasin, 2010; Yan *et al.*, 2007; Zhu *et al.*, 2002; see below).

During B cell differentiation in the bone marrow (or fetal liver), RAG (and consequently the V(D)J recombination reaction) first assembles IgH variable region gene exons in progenitor-B cells. Following the successful assembly and expression (as a μ IgH chain) of an *IgH* V(D)J, RAG is then retargeted to the IgL (Igκ and Igλ) loci in precursor B cells. Following assembly and expression of an IgL chain that associates with the preexisting IgH chain, the resulting B lymphocytes express the complete Ig molecule on their surface and migrate to the periphery where they can be activated to undergo *IgH* CSR, a separate form of *IgH* locus rearrangement (see below). During T cell development in the thymus, developing T cells first undergo V(D)J recombination at the TCRβ, TCRδ, and TCRγ loci in so-called double negative progenitor T cells (Kreslavsky *et al.*, 2010). Successful rearrangement of *TCR*δ and *TCR*γ genes leads to the development of T cells that express a γ/δ TCR; whereas successful rearrangement and expression of *TCR*β genes leads to the subsequent rearrangement of *TCR*α genes and the ultimate development of α/β TCRs and α/β T cells (Kreslavsky *et al.*, 2010). Following assembly of the gene segments leading to TCR expression, T cells do not undergo any further genomic alterations.

Aberrant joining of RAG-initiated DSBs in both Ig and *TCR* loci generally has been linked to the generation of oncogenic translocations associated with immature human and mouse B and T lymphomas and leukemias. The identity of the locus that participates in the translocations also can give information about the developmental stage in which the translocations occurred. For example, the recurrent TCRδ locus translocations found in

ATM-deficient thymic lymphomas indicates that the translocations were initiated in thymocytes at the early double negative developmental stage (Zha *et al.*, 2010). However, oncogenic translocations that are apparently RAG-initiated are also found in certain human mature B cell lymphomas and might either result from the persistence of unrepaired RAG-initiated DSBs through development in the absence of DSB checkpoints (e.g., in association with ATM deficiency; Callén *et al.*, 2007) or from the persistence of translocations that occurred early in development but which were not activated until later when the IgH3′RR became active (Gostissa *et al.*, 2009a; Janz, 2006; see below). Alternatively, they might also be initiated by RAG expression in certain "mature" lymphocyte populations in the periphery (e.g., Wang *et al.*, 2009) (see below).

2.3. IgH class switch recombination (CSR) and somatic hypermutation (SHM)

Surface IgM expressing B lymphocytes migrate to peripheral lymphoid tissues (e.g., spleen) where they can be activated to undergo CSR, which changes the expressed IgH constant region exons from Cμ to one of a set of downstream constant region exons (generically called "CH genes," e.g., Cγ1) (Chaudhuri *et al.*, 2007). In the mouse, there are seven C_H genes imbedded in the 200 kb region downstream of the Cμ gene (Fig. 4.1). Each of the germline C_H genes (except Cδ) is flanked just upstream by long (up to 12 kb), repetitive switch (S) regions. CSR involves the introduction of DSBs into the "donor" S region upstream of Cμ (Sμ) and into a downstream acceptor S region, which are then end-joined to complete CSR. As a consequence, CSR introduces a large deletion of all the sequences between the donor and acceptor junction, including the Cμ gene; thereby, placing a different C_H gene downstream of the expressed V(D)J and allowing the B cell to express Ig with a different *IgH* constant region and effector function (Chaudhuri *et al.*, 2007; Stavnezer *et al.*, 2008).

CSR is initiated by the activation-induced cytidine deaminase (AID) enzyme (Muramatsu *et al.*, 2000) which introduces lesions into S regions that are processed through the co-opted activities of normal cellular DNA lesion repair pathways to generate DSBs (di Noia and Neuberger, 2007). AID is a single-strand DNA-specific cytidine deaminase that is targeted to duplex S regions via transcription. Each S region is preceded by its own transcriptional promoter that is responsive to certain cytokines and activators allowing CSR. This organization allows CSR to be transcriptionally directed to a particular acceptor S region by outside signals from T cells and other immune cells. (Chaudhuri *et al.*, 2007). AID is induced in antigen-activated B cells and is essential not only for introducing DSBs during CSR but also for introducing SHMs into variable region exons in germinal center (GC) B cells (Muramatsu *et al.*, 2007). Exactly how the DSB versus mutational activities of AID are modulated in CSR and SHM is not known; but it likely has some relationship to differences in the S region and variable region exon target sequences.

Not surprisingly, AID activity during the CSR process has been implicated in generating chromosomal translocations (Franco *et al.*, 2006a; Ramiro *et al.*, 2006). Additional evidence has also implicated AID activity during SHM in chromosomal translocation initiation (Dorsett *et al.*, 2007; Pasqualucci *et al.*, 2008). Indeed, the DSB-initiating activity of AID on S regions is so robust that activated B cells in culture can generate sufficient *IgH* locus DSBs, such that well over 50% can be induced to undergo CSR in culture over a several day period (Boboila *et al.*, 2010a; Cheng *et al.*, 2009; Zhang *et al.*, 2010). How the activities of AID are directly primarily to the IgH S regions in B cells activated for CSR remain unknown. During the process of SHM in antigen-activated GC B cells, AID introduces point mutations, and more rarely, small insertions or deletions, in the variable region exons of the *IgH* and *IgL* genes. The mutation frequency is substantial, at about 10^{-3} to 10^{-4} per basepair per generation, allowing the selection of GC B cells with improved antigen specificity and affinity (Li *et al.*, 2004). AID targets so-called RGYW and WRCY motifs, of which AGCT

is a consensus sequence (Rogozin and Kolchanov, 1992); these sequence motifs are abundant both within variable region exons and even more so in S regions (Chaudhuri *et al.*, 2007). However, these motifs alone, even with specific transcription, do not appear sufficient to explain the great specificity of AID for *Ig* loci (Wagner *et al.*, 1995). Despite the great specificity of AID for *Ig* loci, the enzyme has been found to have numerous other targets, albeit at highly reduced efficiency (Liu *et al.*, 2008; Odegard and Schatz, 2006; Robbiani *et al.*, 2009). Indeed, AID can induce the DSBs in both the *Ig*H and *c-Myc* loci that mediate *IgH* to *c-Myc* translocations in activated mouse B cells in culture (Robbiani *et al.*, 2009).

2.4. DNA double strand break (DSB) repair during V(D) J recombination and IgH CSR

There are several known DSB repair pathways in mammalian cells. Following DNA replication, DSBs can be repaired by homologous recombination (HR) using the homologous chromosome as a template for accurate repair (reviewed by Bernstein and Rothstein, 2009; Li and Heyer, 2008; Strathern *et al.*, 1995). HR, as its name implies, requires long stretches of homology to initiate repair. This pathway is not thought to function in G1 phase cells where V(D)J and CSR are initiated. Single-strand annealing (SSA) is also a homology-dependent mode of joining and is closely related to HR (Ivanov *et al.*, 1996; Liang *et al.*, 1998; Lin *et al.*, 1984). SSA mainly occurs at DSBs between two close direct repeats (distances smaller than 25 kb). DNA ends at DSBs are generally processed to 3′-single-stranded tails. Thus, in SSA, direct repeat sequences next to each side of a DSB can anneal afterward and mediate religation, whereas extruded, noncomplementary regions between both homologous sequences are cleaved away. Accordingly, SSA introduces deletions into the repaired product.

In the G1 cell-cycle phase, DSBs appear to be repaired mainly by classical nonhomologous end-joining (C-NHEJ) which is a pathway that joins ends independent of homology (termed “blunt” or “direct” joining) or ends with very short (usually 1–2 bp) overlap (termed microhomology (MH)-mediated joining). In contrast to HR and SSA, C-NHEJ is unlikely to recreate the same sequence that existed prior to damage, unless the ends happen to be cohesive; but it can rejoin ends with minimal loss of sequence (Clikeman *et al.*, 2001; Guirouilh-Barbat *et al.*, 2004; Lin *et al.*, 1999). C-NHEJ operates throughout the cell cycle (Mills *et al.*, 2004; Takata *et al.*, 1998), although perhaps less robustly in S phase (Chen *et al.*, 2005; Lee *et al.*, 1997). In the absence of C-NHEJ factors, ends can also be joined by “alternative” end-joining, a still poorly characterized end-joining pathway or pathways. Because of their relevance to V(D)J and CSR, we will describe in more detail below what is known about C-NHEJ and A-EJ pathways.

2.5. Classical nonhomologous end-joining (C-NHEJ)

The C-NHEJ pathway was initially revealed by the finding that three noncomplementing IR-sensitive Chinese hamster ovary cell mutants were deficient for ability to join RAG-initiated DSBs (Taccioli *et al.*, 1993), ultimately leading to the identification of the Ku80, Xrcc4, and DNA-PKcs genes as C-NHEJ components (Lieber *et al.*, 2004; Rooney *et al.*, 2004b). Subsequently, additional components (e.g., Ku70 which forms a DNA end binding complex with Ku80 and DNA ligase 4 which forms a ligation complex with Xrcc4) were identified based on interactions with known C-NHEJ components and all were shown to function in DNA DSB repair by knockout studies in mice (reviewed by Franco *et al.*, 2006b; Lieber *et al.*, 2004; Rooney *et al.*, 2004b; Zha *et al.*, 2009). Another C-NHEJ factor, Artemis, was identified based on its mutation in certain radio-sensitive SCID patients (Moshous *et al.*, 2001). Most recently, yet another potential C-NHEJ factor, termed Cernunnos or XLF, was also identified based on its mutation in radio-sensitive, immune-deficient patients (Ahnesorg *et al.*, 2006; Buck *et al.*, 2006). It has been shown that XLF/Cernunnos stimulates the

ligation reaction of noncompatible DNA ends *in vitro* (Ahnesorg *et al.*, 2006) and is required for gap filling of partially cohesive ends by polymerase λ or μ (Akopiant *et al.*, 2009). However, unlike all of the other C-NHEJ factors mentioned above, XLF is not required for V(D)J recombination in mice so its precise role in C-NHEJ remains to be fully determined (Li *et al.*, 2008).

C-NHEJ is initiated by binding of the Ku70/Ku80 heterodimer to the broken ends (Fig. 4.2). Ku70/Ku80 forms a ring-like structure that can accommodate double-stranded DNA while serving to protect the ends and recruit other components of the C-NHEJ machinery (Mahajan *et al.*, 2002;Mari *et al.*, 2006;Mickelsen *et al.*, 1999;Walker *et al.*, 2001). The DNA-PK catalytic subunit (DNA-PKcs) bound to DNA with Ku70 and K80 form the fully functional DNA-PK holoenzyme (Falck *et al.*, 2005;Gell and Jackson, 1999;Singleton *et al.*, 1999). Like ATM, DNA-PK belongs to the PIKK (Phosphatidylinositol-3-phosphate-related kinases) family of protein kinases, and once assembled with Ku at a DNA end, DNA-PKcs is activated. One of its main functions in C-NHEJ is to promote synapsing of two broken DNA ends, while exposing them to enzymes that can accomplish necessary modifications prior to ligation (DeFazio *et al.*, 2002;Spagnolo *et al.*, 2006;Yaneva *et al.*, 1997). Recently, *in vitro* studies have indicated that Ku70/80 on its own is a 5 □ -dRP/AP lyase that can act upon an abasic site occurring at double-strand end (Roberts *et al.*, 2010). Other end-modifying functions would include 5 □ –3 □ exonucleolytic trimming by Artemis (Dahm, 2007;Niewolik *et al.*, 2006). Gap filling apparently is accomplished by polymerase μ or λ (Uchiyama *et al.*, 2009). Finally, ligation of the processed DNA ends is mediated by a C-NHEJ factor complex consisting of ligase 4 and its cofactor Xrcc4 (Grawunder *et al.*, 1997).

Ku70, Ku80, Xrcc4, and ligase 4 are conserved NHEJ factors from yeast to mammalian cells and are absolutely required for both the coding and RSS joining reactions during V(D)J recombination; these four factors are considered “core” C-NHEJ factors (Rooney *et al.*, 2004b; Zha *et al.*, 2009). In contrast, the nonevolutionarily conserved factors DNA-PKcs and Artemis, which have major roles in joining ends that require processing, are relatively dispensable for V(D)J RSS joining, but absolutely required to open V(D)J coding end hairpins prior to joining (Lieber *et al.*, 2004; Rooney *et al.*, 2004a). Deficiencies for any of these C-NHEJ factors lead to severe combined immune deficiency due to the inability of progenitor lymphocytes to join V(D)J coding ends (Mills *et al.*, 2003; Rooney *et al.*, 2004b). Indeed, deficiency for Xrcc4 or ligase 4 leads to late embryonic lethality associated with the widespread death of newly differentiated neurons due to a p53-dependent response to unrepaired DSBs, indicating the broad importance of this pathway in repairing DNA damage (Gao *et al.*, 1998). In addition, deficiency for any core C-NHEJ factor, as well as for DNA-PKcs or Artemis, leads to frequent chromosomal translocations in a wide variety of cell types, clearly indicating that C-NHEJ factors are required to suppress translocations and further indicating that, in the absence of C-NHEJ, other repair pathways can robustly generate translocations (Difilippantonio *et al.*, 2000; Ferguson *et al.*, 2000; Gao *et al.*, 2000). Finally, the role of C-NHEJ pathway in suppressing translocations might also reflect the propensity of this pathway to join DSBs within a chromosome, as opposed to joining to DSBs on other chromosomes to generate translocations (Ferguson *et al.*, 2000; Zarrin *et al.*, 2007). How C-NHEJ might be preferentially restricted to intrachromosomal joins is unknown but one hypothesis is that such a restriction might be imposed if C-NHEJ was somehow restricted to an intrachromosomal DSB response (Boboila *et al.*, 2010b), which is also required to prevent DSBs from separating into chromosomal breaks and generating translocations (Franco *et al.*, 2006a,b; see below).

2.6. Alternative end-joining (A-EJ)

Evidence for an alternative end-joining pathway or pathways came from studies that showed C-NHEJ-deficient cell could still fuse DNA ends via end-joining (Boulton and Jackson,

1996; Kabotyanski *et al.*, 1998; Wang *et al.*, 2003; Zha *et al.*, 2009). Indeed, alternative modes of end-joining can be differentiated from C-NHEJ both by biochemical or genetic requirements in various systems (Iliakis *et al.*, 2004; Wang *et al.*, 2006; Yan *et al.*, 2007). Factors including NBS1, Mre11, CtlP, DNA Ligase 3, Parp1, and XRCC1 have been suggested to be components of the A-EJ pathways (Audebert *et al.*, 2004; Deriano *et al.*, 2009; Dinkelmann *et al.*, 2009; Rass *et al.*, 2009; Wang *et al.*, 2005, 2006; Xie *et al.*, 2009), but there is still no agreement as to whether A-EJ pathway is a single or multiple pathways. Indeed, it seems likely that there is more than one form of A-EJ and, to date, the best definition of A-EJ would be any form of end-joining that occurs in the absence of C-NHEJ factors.

End-joining activities that occur in the absence of C-NHEJ have been demonstrated in experiments where naked linear DNA with various end structures is transfected into C-NHEJ deficient cells. Notably, cells deficient in DNA-PKcs (Chang *et al.*, 1993; Harrington *et al.*, 1992), Ku (Kabotyanski *et al.*, 1998), Xrcc4 (Kabotyanski *et al.*, 1998), or DNA ligase 4 (Verkaik *et al.*, 2002) were able to carryout linear DNA recircularization via end joining. Likewise, DSBs introduced at chromatinized genomic locations were also repaired in NHEJ-deficient cells (Guirouilh-Barbat *et al.*, 2004; Rass *et al.*, 2009; Xie *et al.*, 2009). Further, it has been found that CSR can be catalyzed at levels approaching 50% of those in WT cells in the absence of any of the core C-NHEJ factors (Boboila *et al.*, 2010a; Han and Yu, 2008; Yan *et al.*, 2007). One possibility is that in the absence of one C-NHEJ component, C-NHEJ proceeds but at reduced levels perhaps by some other factor filling in for the missing component (e.g., DNA Ligase 1 or Ligase 3, substituting for Ligase 4). However, it was recently found that CSR can still occur at similar levels in B cells deficient in both Ku70 and DNA ligase 4, the DSB recognition and joining components of C-NHEJ. These latter findings provide the strongest evidence to date that A-EJ is a totally distinct pathway (or pathways) from C-NHEJ. Notably, A-EJ does not function at all in V(D)J recombination, which is completely reliant on C-NHEJ for junction formation. This restriction is mediated by RAG which shepherds the V(D)J recombination reaction specifically into C-NHEJ and excludes other repair pathways (Corneo *et al.*, 2007).

During both V(D)J recombination and CSR, C-NHEJ generates direct junctions and junctions with short MH at relatively similar levels (Boboila *et al.*, 2010a; Komori *et al.*, 1993; Yan *et al.*, 2007). Notably, in several of the systems outlined above, A-EJ was associated with the increased usage of MH-mediated end-joining. Thus, in Xrcc4 or Ligase 4-deficient cells, CSR junctions were essentially all MH-mediated and the MHs were on average longer than those associated with C-NHEJ. However, the frequent usage of MH by A-EJ may be manifested predominantly in situations where the substrate, such as S regions, readily provides long MH. In other cases, for example joining of chromosomally integrated I-SceI substrates, a substantial proportion of joins in Ku or Ligase 4-deficient cells were direct (Guirouilh-Barbat *et al.*, 2004). Again, as outlined above, the most useful current definition of A-EJ is end-joining not reliant on large stretches of homology (e.g., as required by SSA) that occurs in the absence of C-NHEJ components.

The relevance for C-NHEJ in suppressing oncogenic translocations in mice is well established as C-NHEJ-deficient mice (whether they lack Ku80, Xrcc4, Ligase 4, DNA-PKcs, or Artemis) that are also deficient for p53 routinely develop pro-B cell lymphomas harboring oncogenic chromosomal translocations involving the *IgH* and *c-Myc,* or in the case of Artemis deficiency, *N-myc* genes (Difilippantonio *et al.*, 2000; Frank *et al.*, 2000; Gao *et al.*, 2000; Rooney *et al.*, 2004a; Zhu *et al.*, 2002). Notably, Xrcc4 or Ligase 4-deficient progenitor-B cells, when also deficient for the p53 checkpoint protein, give rise to lymphomas with RAG-dependent *IgH* translocations in the vicinity of *c-Myc*, all of which are catalyzed by A-EJ and have MH-mediated translocation junctions (Zhu *et al.*, 2002).

This finding led to the first suggestion that A-EJ may be a translocation-prone pathway (Zhu *et al.*, 2002). Similarly, in C-NHEJ-deficient activated B cells, A-EJ frequently joins *IgH* locus breaks to other chromosomes to generate translocations, including *IgH* to *c-Myc* translocations that also appear as oncogenic translocations in p53-deficient B cells (Wang *et al.*, 2009; Yan *et al.*, 2007). Likewise, p53-deficient mice that lack Xrcc4 in the central nervous system routinely develop medulloblastomas with recurrent translocations (Yan *et al.*, 2006).

A recent report indicated that Ligase 4 and Xrcc4 repress the formation of translocations (Simsek and Jasin, 2010), and found no difference in the fine structure of the translocation junctions formed in their model system with or without Ligase 4 or Xrcc4. This is consistent with the idea that A-EJ is the major pathway mediating chromosomal translocations in the absence of C-NHEJ (Boboila *et al.*, 2010b; Wang *et al.*, 2008; Yan *et al.*, 2007). Analyses of chromosomal translocation junctions also led to the suggestion that A-EJ might predispose to translocations in humans (e.g., Zhang and Rowley, 2006). The reason for frequent A-EJ-catalyzed translocations in the absence of C-NHEJ is not well understood. It could be that this pathway is simply not as biased to forming intrachromosomal joins as is C-NHEJ (Boboila *et al.*, 2010b). Alternatively, it may be that the apparently high frequency of translocations catalyzed by A-EJ simply reflects the fact that more DSBs are not rapidly repaired in the absence of C-NHEJ and, therefore, persist as substrates for A-EJ (Yan *et al.*, 2007).

2.7. DSB response factors

Upon acquisition of a DSB in the genome, the DSB response is activated and DSB response factors are recruited to the broken DNA ends where they activate signal pathways that lead to cell-cycle arrest, initiate repair of the broken DNA ends, and induce apoptosis or senescence if the repair of DSBs cannot be achieved (Sancar *et al.*, 2004). Of particular relevance to the V(D)J and CSR processes, ATM is activated upon formation of DNA DSBs in the G1 phase of the cell cycle (Abraham, 2001; Canman *et al.*, 1998). During the DSB response, ATM phosphorylates and activates p53, which monitors DSBs in the context of G1 and G2/M checkpoints and signals cell-cycle arrest or apoptosis (Vogelstein *et al.*, 2000). ATM also phosphorylates additional substrates that are key in cell-cycle checkpoint responses, in recruiting or regulating repair factors, and in immobilizing broken DNA ends prior to repair; these substrates include histone H2AX, MDC1, and 53BP1 (Bassing and Alt, 2004).

The histone variant H2AX is one of the most conserved H2A variants across species (Kinner *et al.*, 2008). Upon formation of a DSB, H2AX molecules in nucleosomes flanking DSBs are phosphorylated on serine 139 to form γ-H2AX (Rogakou *et al.*, 1998). The phosphorylation event is initially localized to the site of the DSB but then spreads to a large region (up to 100 kb) around the DSB (Rogakou *et al.*, 1998, 1999; Savic *et al.*, 2009). γ-H2AX carries out its DSB response functions by signaling DSBs and forming a platform for the recruitment of multiple proteins of the DSB response pathway to form large γ-H2AX foci (Kinner *et al.*, 2008). Although evidence suggests that MDC1 is a main interaction partner for γ-H2AX in higher eukaryotes, other interaction partners (e.g., NBS1, 53BP1) have been described (Kobayashi *et al.*, 2002; Stewart *et al.*, 2003; Ward *et al.*, 2003), and are recruited to γ-H2AX foci. Phosphorylated serine 139 on H2AX plays a role as an anchor residue for protein–protein interactions, apart from which it may also serve to promote open chromatin structures due to its negative charge. It has been observed that local chromatin density decreases after generation of DSBs (Downs *et al.*, 2000).

DSB response factor deficiencies lead to unrepaired chromosomal breaks and translocations. For example, ATM, H2AX, and 53BP1 are needed to prevent *IgH* locus DSBs generated by

AID from progressing to chromosomal breaks and translocations, including oncogenic I*gH/c-Myc* translocations (Franco *et al.*, 2006a; Ramiro *et al.*, 2006). In addition, H2AX deficiency combined with p53 deficiency leads to T and B cell lymphomas that harbor clonal translocations; in B cells, often recurrent *IgH* to *c-Myc* translocations (Bassing *et al.*, 2003; Celeste *et al.*, 2003). These findings led to the proposal that DSB response foci tether chromosomal DSBs to promote C-NHEJ joining within a chromosome and prevent translocations (Bassing and Alt, 2004; Bassing *et al.*, 2003; Boboila *et al.*, 2010b; Franco *et al.*, 2006b; Zarrin *et al.*, 2007). Although deficiency in any one of the described DSB response factors increases genomic instability and reduces CSR, the extent varies for the different factors. The highest levels of genomic instability and chromosomal translocations are associated with ATM deficiency and the most severe CSR defect is observed in 53BP1-deficient cells, the latter possibly suggesting CSR-specific functions of 53BP1 beyond those served by the DSB response *per se* (Franco *et al.*, 2006a; Ramiro *et al.*, 2006). ATM and, to a lesser extent, 53BP1 are also required for fully normal joining of chromosomal DSBs during V(D)J recombination (Bredemeyer *et al.*, 2006; Difilippantonio *et al.*, 2008).

53BP1 also has been proposed to play a role in regulating the mobility of DNA ends, in particular at deprotected telomeres (de Lange, 2009), possibly indicating that 53BP1 influences chromatin dynamics and facilitates repair of breaks at distant sites (Difilippantonio *et al.*, 2008). DSB response factors that are involved in tethering of broken DNA ends also include NBS1, MRE11, and Rad50, which together comprise the MRN complex. In addition to its function in tethering broken DNA ends, the MRN complex is involved in many cellular processes such as DNA replication, DNA repair, cell cycle checkpoints, and telomere maintenance (D'Amours and Jackson, 2002; Van den Bosch *et al.*, 2003). Genetic studies have suggested a role for the MRN complex in both C-NHEJ and A-EJ during CSR (Deriano *et al.*, 2009; Dinkelmann *et al.*, 2009; Rass *et al.*, 2009; Xie *et al.*, 2009).

3. MECHANISTIC FACTORS THAT INFLUENCE THE APPEARANCE OF CHROMOSOMAL TRANSLOCATIONS

3.1. Overview

Recurrent translocations in tumors generally are thought to arise spontaneously as low-frequency events that are selected at the cellular level for contribution to oncogenesis (Fig. 4.3). Thus, underlying mechanistic features that influence the generation of well-studied translocations may be masked. While selection for oncogenic activities is clearly a key factor in the appearance of oncogenic translocations, recent studies have shown that mechanistic factors also are basic to the process and can influence appearance of sequences, including cellular oncogenes, in recurrent translocations (Gostissa *et al.*, 2009a,b;Wang *et al.*, 2009). Mechanistic factors that might influence translocations include DSB frequency at translocating loci and factors that influence such DSBs (Robbiani *et al.*, 2008;Wang *et al.*, 2009), factors that contribute to two translocating loci lying in close enough proximity in the interphase nucleus to be joined (Meaburn *et al.*, 2007;Wang *et al.*, 2009), and mechanisms that circumvent functions of the cellular DSB response and repair pathways that promote joining of DSBs within a chromosome and suppress joining of DSBs between chromosomes (Ferguson *et al.*, 2000;Franco *et al.*, 2006a,b;Ramiro *et al.*, 2006).

3.2. Role of mechanistic factors in promoting chromosomal translocations

3.2.1. Chromosomal position—Accumulating evidence suggests that the genome of eukaryotes is not distributed randomly in the interphase nucleus but rather follows a distinct spatial order (Lanctô t *et al.*, 2007; Lieberman-Aiden *et al.*, 2009; Meaburn *et al.*, 2007). Importantly, spatial genome organization is highly tissue- and cell-type specific and is

affected by transcriptional activity, cellular proliferation status, and possibly many pathways (Bridger *et al.*, 2000; Cremer *et al.*, 2003; Parada *et al.*, 2004). The defined spatial localization of a chromosome within the nucleus is referred to as chromosome territory (Cremer and Cremer, 2001; Foster and Bridger, 2005; Parada and Misteli, 2002).

Chromosomal position of two, otherwise, relatively equivalent oncogenes, can greatly influence their ability to contribute to oncogenic translocations. Thus, the *c-myc* and *N-myc* cellular oncogenes belong to the same family, have similar functional activity *in vivo* including oncogenic potential, and are expressed in developing lymphocytes and neuronal cells (Kohl *et al.*, 1983; Malynn *et al.*, 2000; Zimmerman *et al.*, 1986). Yet, translocations in B cell lymphomas, such as the pro-B lymphomas that arise from Xrcc4/p53 deficient developing mouse B cells or in human Burkitt's lymphomas (Küppers and Dalla-Favera, 2001), frequently involve *c-myc* but not *N-myc* (e.g., Difilippantonio *et al.*, 2000; Zhu *et al.*, 2002); while *N-myc,* but not *c-myc,* is amplified in human neuroblastomas (e.g., Kohl *et al.*, 1983) and mouse neuroblastomas that arise from Xrcc4/p53 deficient developing neuronal cells (Yan *et al.*, 2006). However, when inserted in place of *c-myc* coding sequence, *N-myc* coding sequences compete well with the normal *c-myc* allele to generate recurrent translocations leading to pro-B cell lymphomas (Gostissa *et al.*, 2009b). Thus, these gene replacement studies rule out cellular selection as the only factor that promotes recurrent *c-myc* but not *N-myc* translocations in developing B lineage cells and clearly demonstrate that chromosomal location can markedly influence ability of a cellular oncogene to contribute to recurrent translocations. In this context, chromosomal environment might influence the frequency of DSBs around a potential oncogene, influence its spatial proximity to a translocation partner, alter its chromatin struction or transcriptional regulation, or predispose it to a particular form of DNA repair.

Recent studies have provided a detailed picture of the organization of the genome within the mammalian nucleus that is based on nonrandomly positioned genes and chromosomes (e.g., Lieberman-Aiden *et al.*, 2009). In this context, there are two long-standing models for translocation initiation (Fig. 4.4). The "contact-first" model postulates that translocations are restricted to DSBs that arise in proximally positioned regions of the genome, while the "breakage-first" model suggests DSBs initially located far apart can move together (Meaburn *et al.*, 2007). It should be noted that the breakage- and contact-first models, while extremes, are not necessarily mutually exclusive. In any case, the spatial disposition of chromosomes (i.e., the relative positioning of chromosomal domains or whole chromosomes with respect to each other) likely impacts substantially on patterns of genome rearrangements. Numerous studies have indicated that loci that are proximal to each other in 3D appear to have increased likelihood of engaging in a translocation event (reviewed by Meaburn *et al.*, 2007). Imaging studies have demonstrated that certain test DSBs are relatively immobile in the nucleus (Soutoglou *et al.*, 2007). However, the possibility that some DSBs on distant chromosomes are brought together (synapsed) for repair after their generation, as has been reported for DSBs in yeast cells (Lisby *et al.*, 2003), has not been ruled out and awaits further experimentation.

3.2.2. DSBs—Despite the key role of DSBs in translocations and genomic instability, a unified understanding of the elements that predispose to DSBs in many tumors is still lacking. In this regard, it is also worth reemphasizing increased levels of particular DSBs could come either by their increased generation or by their decreased repair.

As outlined above, in lymphomas, many oncogenic translocations arise from joining of DSBs in antigen receptor loci to DSBs located in or around a potential oncogene. In developing B and T lymphocytes, RAG can generate *IgH* DSBs that lead to oncogenic translocations by joining to either RAG-generated or non-RAG-generated DSBs elsewhere

in the genome (Fig. 4.5) (e.g., Difilippantonio *et al.*, 2002;Gladdy *et al.*, 2003;Zhu *et al.*, 2002). In mature B cells, AID plays a key role in generating DSBs including *IgH* breaks that lead to high levels of translocations in DNA repair and DSB response deficient backgrounds (Franco *et al.*, 2006a;Ramiro *et al.*, 2006;Wang *et al.*, 2009) and *IgH* DSBs that underlie oncogenic *IgH/c-myc* translocations in mouse lymphoma models (Ramiro *et al.*, 2004). As mentioned above, RAG-initiated DSBs are handled differentially from most DSBs, as RAG proteins channel their joining exclusively into C-NHEJ (Corneo *et al.*, 2007). CSR breaks, on the other hand, are handled much like general cellular DSBs in that they require the DSB response for normal joining over the 100–200 kb distance involved in CSR and are less dependent on C-NHEJ. Thus, CSR DSBs might be considered a model for "intrachromosomal translocations." Indeed, AID-initiated *IgH* locus DSBs can be efficiently joined to yeast I-SceI endonuclease-generated DSBs to cause *IgH* deletions leading to class switching (Zarrin *et al.*, 2007) or to chromosomal translocations (Robbiani *et al.*, 2008;Wang *et al.*, 2009).

DSBs in translocation partners other than antigen receptor loci might be caused by intrinsic factors, such as oxidative metabolism and replication stress, or extrinsic factors such as ionizing radiation or chemotherapeutic agents (Hoeijmakers, 2009). Another source of localized DSBs in most cells types is chromosome fragile sites (Durkin and Glover, 2007). However, the mechanisms that predispose to fragile site formation have not been fully elucidated. In addition, unusual DNA sequence and structures have also been implicated in generating DSBs and translocations (Zhao *et al.*, 2010). On the other hand, AID can generate "off-target" DSBs in *c-Myc* that are joined to AID-initiated *IgH* breaks to form *IgH/c-Myc* translocations like those found in Burkitt's lymphoma (Robbiani *et al.*, 2008) (Fig. 4.5). In addition, recent studies suggested a potential collaboration between AID and RAG in initiating DSBs at nonclassical RAG target sites in developing B cells, thereby predisposing to oncogenic translocations found in human acute lymphoblastic leukemias (Tsai *et al.*, 2008). Notably, transgenic overexpression of AID in B cells leads to rather widespread genome instability (Robbiani *et al.*, 2009). Identifications of such AID "off-targets" should provide important insights into the targeting of chromosomal translocations in cancers derived from activated B cells.

Surprisingly, AID has been suggested to be involved in the generation of chromosomal translocations and mutations in nonlymphoid tumors (Lin *et al.*, 2009; Okazaki *et al.*, 2007). In this context, AID has been reported to be expressed in embryonic stem cells, where it is proposed to have a role in demethylation of 5-methylcytosine residues and, thereby, a role in epigenetic regulation (Bhutani *et al.*, 2010). AID has also been implicated in gastric cancers and in recurrent translocations in prostate cancers (Lin *et al.*, 2009; Matsumoto *et al.*, 2007). Thus, further understanding of the role of AID in destabilizing the genome of cells outside the immune system is needed.

3.2.3. Chromosomal features that promote selection—Chromosomal translocations can be subject to various negative and positive selection events in dividing cells. As discussed above, "driver" translocations are causally implicated in the initiation of oncogenesis via positive selection for a conferred growth advantage of the cancer progenitor cell in the microenvironment of the tissue in which it arises. In this regard, most initial chromosome translocation events are indeed quite rare. For example, *IgH/c-Myc* translocations generally appear to occur in less than 1 in 10^6 activated mouse B cells (Ramiro *et al.*, 2006; Wang *et al.*, 2009), consistent with strong oncogenic selection playing a major role in their recurrent appearance in mature B cell lymphomas.

Chromosomal features of particular loci beyond their propensity to break or their spatial proximity to translocation partner loci can play a role in the appearance of recurrent

oncogenic translocations. Thus, transgenic mice bearing *IgH* intronic enhancer (iEμ) or IgH3′RR sequences fused to *c-Myc*, respectively, are predisposed to the development of early and mature B lineage lymphomas, demonstrating that both elements can confer oncogenic *c-Myc* expression (Adams *et al.*, 1985; Schmidt *et al.*, 1988; Truffinet *et al.*, 2007). However, in most mature B cell lymphomas that arise as a result of errors in *IgH* CSR (such as certain Burkitt's lymphomas), *IgH/Myc* translocations delete iEμ and place *c-Myc* up to 200 kb upstream of the IgH3′RR (Janz, 2006; Küppers and Dalla-Favera, 2001). However, recent studies have shown that the IgH3′RR, which was previously shown to regulate transcription of C_H gene promoters in the context of CSR over long distances (Cogné *et al.*, 1994), can also activate expression of the *c-myc* oncogene over 200 kb distances and, as such, is required for development of tumors in a mouse model for peripheral B cell lymphomas. (Gostissa *et al.*, 2009a) (Fig. 4.5). Thus, the IgH3′RR is a locus-specific element that plays a critical role in driving the expression of oncogenic *IgH/c-Myc* translocation in mature B cell lymphomas. Indeed, it is possible that the iEμ element does not play a major role in activating translocated oncogene expression even in *IgH* locus translocations in which it is retained (Janz, 2006; Gostissa *et al.*, 2009a). Finally, other recent studies have shown that deletion of a 2.2 kb promoter region of *c-myc* gene severely impairs its transcription and drastically reduces the frequency of *IgH/Myc* translocations in peripheral B cells (Robbiani *et al.*, 2008). It seems likely that transcriptional regulatory elements in other translocation target loci may similarly play a role in promoting the occurrence of oncogenic translocations.

3.2.4. DNA repair—The potential roles of DNA DSB response and repair pathways in the suppression of translocations have been discussed above. In this context, it is notable that the vast majority of translocation junctions mapped in tumors shows typical characteristics of C-NHEJ or A-EJ (e.g., junctions with no or limited microhomology as well as deletions, insertions, and end modifications) (Gillert *et al.*, 1999; Zhang and Rowley, 2006). These observations are in accordance with studies in model systems revealing extensive use of C-NHEJ or A-EJ in translocation formation (Weinstock *et al.*, 2006a,b). Of note, in model systems, NHEJ-mediated translocation junctions exhibit more extensive use of deletions, insertions, and micro-homologies than junctions of standard NHEJ-repaired DNA DSBs (Weinstock *et al.*, 2006a,b). Tumor patient-derived junctions of reciprocal translocations show comparable patterns (Stephens *et al.*, 2009; Weinstock *et al.*, 2006a), thus, suggesting the existence of potential mechanistic differences.

3.3. Recurrent "passenger" translocations promoted by multiple mechanistic factors

Xrcc4-deficient B cells very frequently translocate RAG-initiated DSBs in the *Igλ* locus on chromosome 16 to AID-initiated *IgH* breaks on chromosome 12, likely because the two loci are both frequently broken in the same cells and because they frequently lie in close spatial proximity, and potentially because they are joined by A-EJ. Notably, colocalization of *Igλ* and *IgH* in these cells, as measured by 3D interphase FISH, was relatively focal with respect to the position of *Igλ* on chromosome 16, implicating aspects of particular chromosomal regions, as opposed to broader chromosomal territories, as important in determining spatial proximity and translocation frequency (Wang *et al.*, 2009). Strikingly, these mechanistic factors led to such a high frequency of *Igλ* to *IgH* locus translocations in progenitors of Xrcc4/p53 double-deficient B cell lymphomas, that they appear as recurrent translocations in the tumors apparently without conferring any known selective advantage. This finding highlights the potential role of mechanistic factors in helping to promote translocations.

4. PERSPECTIVE

Thus far, most studies of translocation mechanisms have been focused on and limited to a few specific translocations, namely those observed as recurrent translocations in tumors. Although studies from tumor models have provided enormously useful insights on the mechanistic factors involved in chromosomal translocations, new experimental systems will be necessary to advance such studies. Thus, in tumor studies, strong *in vivo* oncogenic selection for translocation products could have masked the identification of factors influencing early translocation steps. A more representative system to study translocation-initiating events should minimize cellular selection. Recently, work on primary B cells, A-MuLV transformed pro-B cell lines, ES cell lines, and prostate cancer cell lines (Ramiro *et al.*, 2006; Wang *et al.*, 2009; Mahowald *et al.*, 2009; Weinstock *et al.*, 2008; Lin *et al.*, 2009) has provided some direction into the requirements for establishing experimental systems to study the initiation phase of the translocations. In addition, cancer genome studies have provided further insights and suggested that there are more rearrangements in tumors than previously expected (Stephens *et al.*, 2009; Stratton *et al.*, 2009). Also, studies on primary B cells activated for CSR suggested that 1–5% of the metaphases harbor *IgH*-related chromosomal translocations in DSB response and DSB repair deficient backgrounds (Franco *et al.*, 2006a,b; Wang *et al.*, 2009; Yan *et al.*, 2007). Most of these *IgH* locus translocations involve unidentified partners, raising the potentialy tractable question of determining the unselected pattern of *IgH* locus translocations from a genome-wide viewpoint. To further our understanding of the role of various mechanistic factors in promoting translocations, future studies should focus on the development of methods to study these events genome-wide in various cell types and tumor progenitors, an effort that should benefit greatly from the rapid evolution of the genomic tools, especially high-throughput sequencing techniques.

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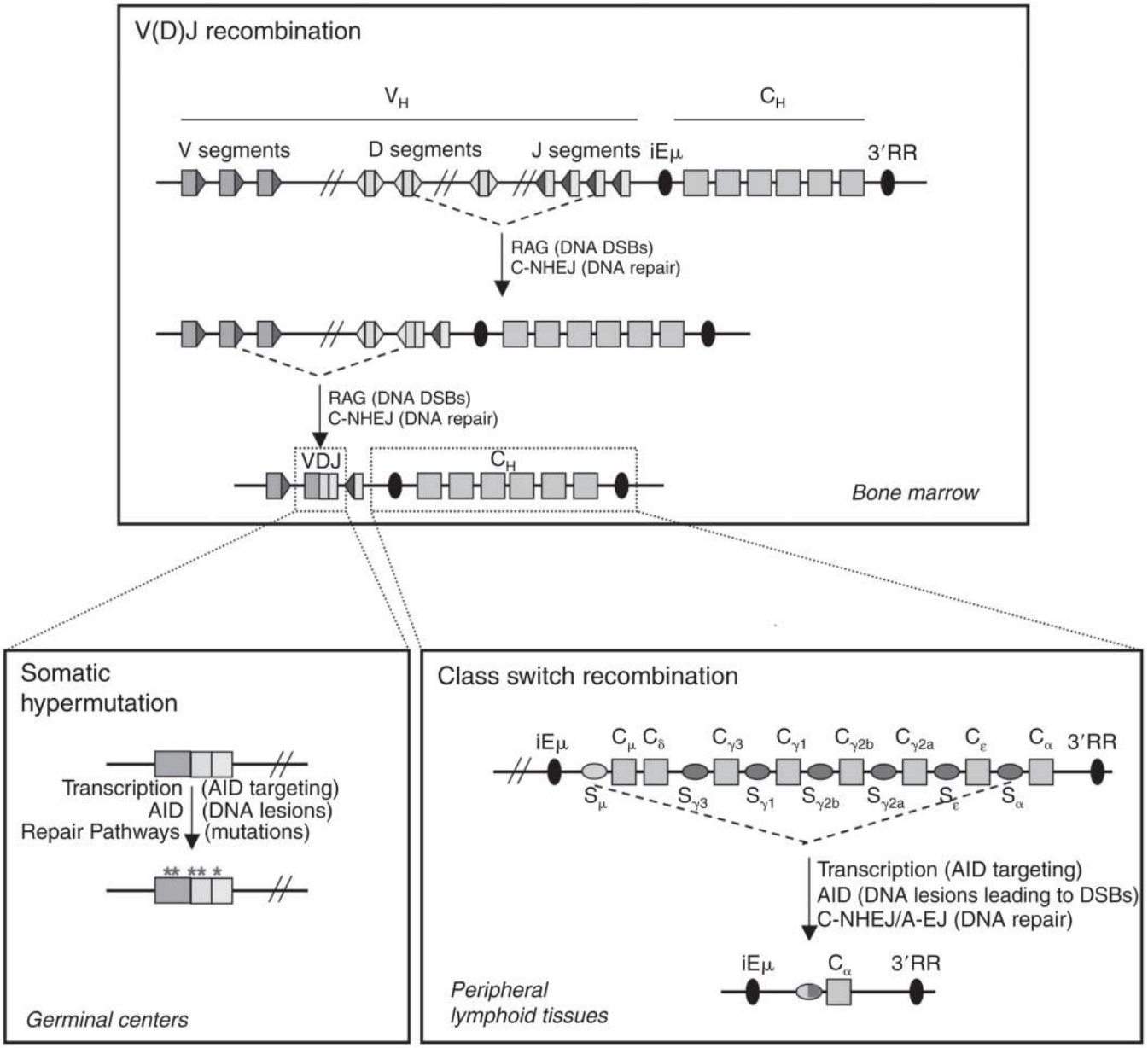

FIGURE 4.1.

DNA rearrangement events in the *IgH* locus during B cell development. (See Text for details.)

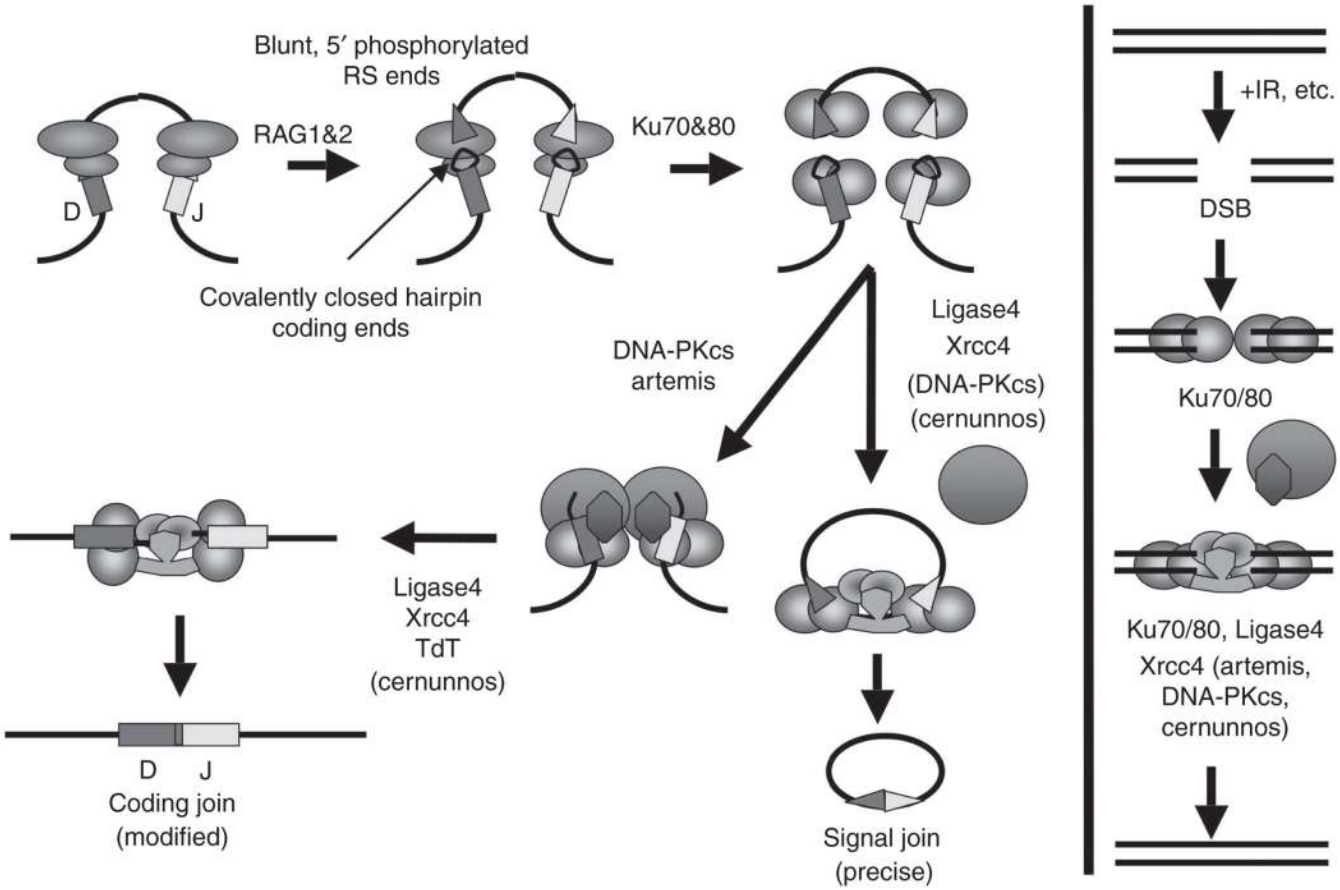

FIGURE 4.2.
Classic nonhomologous end joining in V(D)J recombination and general double-strand break repair. (Adapted from Dudley *et al.*, 2005). (See text for details.)

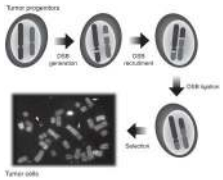

FIGURE 4.3.

Formation of recurrent reciprocal chromosomal translocations in tumors. First, two DSBs occur at two nonhomologous chromosomes. Then the four broken ends, after escape from normal DSB repair, are synapsed and ligated to form reciprocal chromosomal translocations. Finally, recurrent translocations in tumors are generally thought to arise spontaneously as very low-frequency events that are strongly selected at the cellular level via their contributions to oncogenesis.

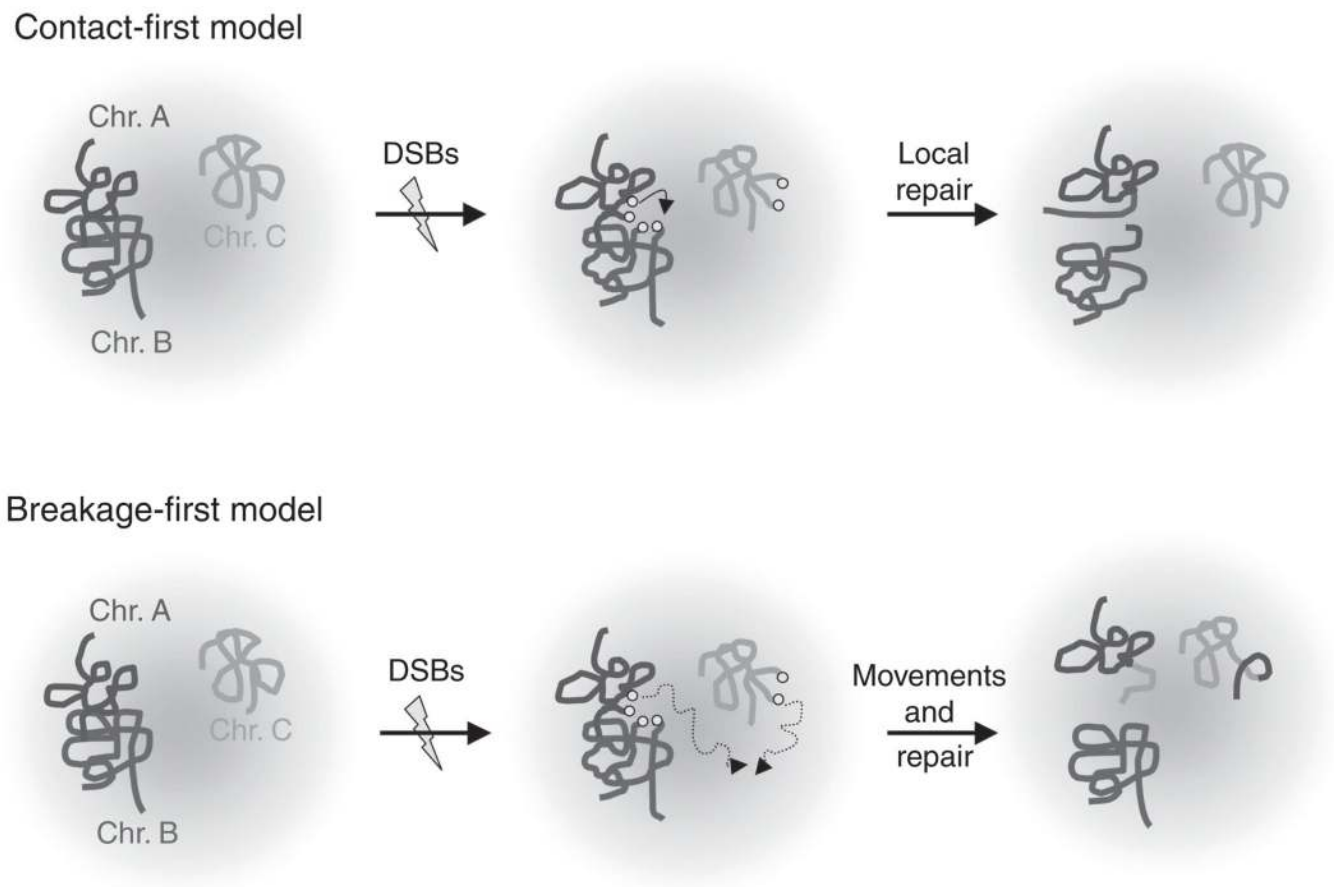

FIGURE 4.4.
The "contact-first" and "breakage-first" models for chromosomal translocation. (See text for details).

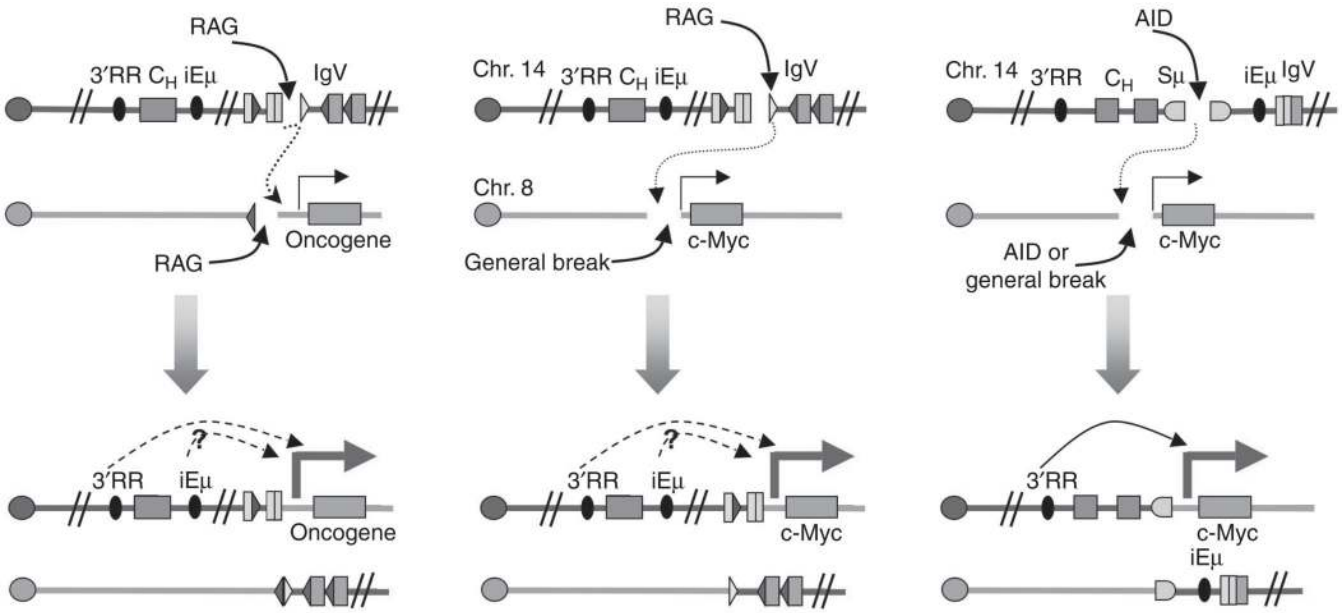

FIGURE 4.5.

Chromosomal translocations involving aberrant V(D)J recombination and CSR. Left: aberrant V(D)J recombination between RSS in the *IgH* locus and cryptic RSS in a different chromosome. Middle: Formation of reciprocal translocations between RAG-generated and non-RAG-generated DSBs. In both these cases, the iEμ and IgH3′RR are both linked to c-myc and can potentially contribute to its over-expression (dotted arrows). Right: Formation of reciprocal translocations between AID-generated breaks at the *IgH* and *c-Myc* loci; only the IgH3′RR is present in this configuration and it has been shown to enhance c-myc expression (solid arrow). (see text for further details.)