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Mechanisms of Programmed DNA Lesions and Genomic Instability in the Immune System

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Abstract

Chromosomal translocations involving antigen receptor loci are common in lymphoid malignancies. Translocations require DNA double-strand breaks (DSBs) at two chromosomal sites, their physical juxtaposition, and their fusion by end joining. Ability of lymphocytes to generate diverse repertoires of antigen receptors and effector antibodies derives from programmed genomic alterations that produce DSBs. We discuss these lymphocyte-specific processes, with a focus on mechanisms that provide requisite DSB target specificity and mechanisms that suppress DSB translocation. We also discuss recent work that provides new insights into DSB repair pathways and influences of three-dimensional genome organization on physiological processes and cancer genomes.

Introduction

The B cell antigen receptor (“BCR”) is comprised of immunoglobulin (Ig) heavy (IgH) and light (Igκ or Igγ, collectively called IgL) chains. The T cell antigen receptor (TCR) is highly related. The N-terminal variable region of Ig and TCR chains binds antigen and is encoded by germline V, D, and J gene segments, which are assembled into V(D)J exons in developing B and T lymphocytes (Cobb et al., 2006; Krangel, 2009). V(D)J recombination contributes to diverse antigen receptor repertoires by assembling the numerous Vs, Ds and Js in different combinations (Davis and Bjoerkman, 1988). Transcription from a V promoter runs through the assembled V(D)J exon and several downstream exons that encode a C-terminal “constant” region. Upon antigen activation, mature B cells undergo two additional genomic alterations. Somatic hypermutation (SHM) introduces point mutations into *IgH* and *IgL* variable region exons, allowing selection of B cells that produce higher affinity antibodies (di Noia and Neuberger, 2007). *IgH* class switch recombination (CSR) replaces one set of *IgH* constant region exons (C_Hs) with another, allowing B cells to secrete different effector antibody classes containing the variable region that contributed to their

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BCR (Chaudhuri et al., 2007). In both mice and humans, antigen receptor loci lie on different chromosomes or distal parts of a given chromosome, spanning large distances. Antigen receptor loci also contain strong regulatory regions that contribute to both assembly and expression of Ig and TCR genes (Cobb et al., 2006; Krangel, 2009).

The V(D)J recombination process has distinct cutting and joining components, rather than being carried out by a single "recombinase". The lymphocyte-specific RAG endonuclease ("RAG"), comprised of Recombination Activating Gene 1 and 2 proteins, initiates the reaction by introducing DSBs adjacent to target Vs, Ds, and Js, which are collectively referred to as "coding segments" (Schatz and Baltimore, 2004). These DSBs are fused to complete V(D)J recombination by classical non-homologous end-joining (C-NHEJ), a major general cellular DSB repair pathway (Boboila et al., 2012). CSR also has distinct initiating and joining components. Activation Induced Cytidine Deaminase ("AID"), which initiates both CSR and SHM, is required for generation of DSBs in downstream portions of *IgH* et al., 2012). Such DSBs are joined, largely, by C-NHEJ to complete CSR (Boboila et al., 2012). Generation of functional antigen receptor loci via DSB intermediates poses great oncogenic risks, as DSBs initiate chromosomal translocations (Zhang et al., 2010). This risk is compounded by the ability of antigen receptor locus regulatory elements to activate translocated oncogene expression (Janz, 2006). Thus, V(D)J recombination-associated DSBs in antigen receptor loci lead to oncogenic translocations found in human B or T cell acute lymphoblastic leukemias (i.e., "B-ALLs" and "T-ALLs") that arise from developing lymphocytes, as well as translocations found in more mature B cell lymphomas; while CSR-associated DSBs give rise to *IgH* translocations found in human mature B cell lymphomas and multiple myeloma (Gostissa et al., 2011; Robbiani and Nussenzweig, 2012). Translocations that fuse the *c-myc* oncogene to *IgH* occur in many human Burkitt's lymphomas and have provided classic examples of both RAG- and AID-initiated translocations (Gostissa et al., 2011; Robbiani and Nussenzweig, 2012). Despite these risks, lymphoid tumors with antigen receptor locus translocations occur only in a small fraction of individuals, as checkpoints eliminate most cells with unrepaired DSBs or oncogenic translocations (Lowe et al., 2004). In addition, risks are further minimized by strict regulation of the formation of antigen receptor locus DSBs and processes that join them.

Mechanism and Control of V(D)J Recombination

V(D)J Recombination Initiation

RAG was discovered based on ability to confer V(D)J recombination to non-lymphoid cells (Schatz and Baltimore, 2004). RAG generates DSBs between two coding segments and short recombination signal sequences (RSSs) that flank them and, then, holds these ends in a post-cleavage synaptic complex (Figure 1A and B). Subsequently, the two coding segment ends are joined to form "coding joins" (Figure 1C,D) and the two RSS ends are joined to form "RSS joins" (Figure 1C,E) (Schatz and Swanson, 2011). In this way, the V(D)J recombination process provides an inherent chromosomal directionality to V(D)J recombination that is dependent on relative orientation of participating gene segments (Bassing et al., 2002). RAG restricts joining to C-NHEJ, with other DSB repair pathways excluded by RAG2 (Corneo et al., 2007). V(D)J recombination occurs in the G1 cell-cycle

phase, ensured by rapid RAG2 degradation at the G1/S transition (Desiderio, 2010). Limiting the reaction to G1 solidifies repair pathway choice and facilitates higher-order regulation. While truncated RAGs have robust transposase activity, reflecting their evolutionary origin, these activities are suppressed in the context of full-length RAGs (Chatterji et al., 2004). RSSs are comprised of a palindromic heptamer and an AT-rich nonamer separated by spacers of 12 or 23 base pairs (referred to as "12RSSs" and "23RSSs"). Joining two coding segments follows the "12/23 rule"; to be cut by RAG, they must be flanked, respectively, by "complementary" 12- and 23RSSs (Schatz and Swanson, 2011; Figure 1). The 12/23 restriction helps guide V(D)J recombination. For example, all V_Hs are flanked by 23RSSs, D_Hs on both sides by 12RSSs, and J_Hs by 23RSSs, preventing direct V_H to J_H joining and necessitating formation of a full V(D)J exon. A beyond 12/23 ("B12/23") restriction also guides joining. TCR Vβs are flanked by 23RSSs, Dβs by 12- and 23RSSs, and Jβs by 12RSSs. Yet, Vβs do not join directly to Jβs, but can if a Jβ 12RSS is replaced with a Dβ 12RSS (Schatz and Swanson, 2011). The 12/23 and, potentially, B12/23 restrictions help minimize "off-target" RAG activity by limiting cleavage to sequences flanked by complementary RSS pairs. This is important because functional RSSs vary from the consensus; allowing abundant genomic representation of "cryptic" RSSs (Tsai and Lieber, 2010).

Developmental Regulation of V(D)J Recombination

RAG expression and V(D)J recombination occur exclusively within lymphocyte lineages and primarily in developing lymphocytes, largely limiting RAG- initiated genomic instability to these cells. Within developing lymphocytes, V(D)J recombination is regulated by higher-order mechanisms to ensure faithful and representative cleavage and joining of widely-separated Vs, Ds, and Js (Cobb et al., 2006). Most broadly, assembly of complete Ig and TCR variable region exons is "lineage specific", B- and T-lineage cells, respectively. Within each lineage, V(D)J recombination is further regulated to ensure diverse and specific repertoires and prevent undesired rearrangements. For the major α/β T cell lineage, relationships between V(D)J recombination and development are similar to those of B cells (Bassing et al., 2002; Krangel, 2009). Therefore, we focus mainly on B cell development and *IgH*, which undergoes all forms of mammalian antigen receptor gene diversification.

Productive *IgH* and *IgL* variable region exon assembly, leading to generation of IgH and IgL chains, directs B cell developmental progression (Figure 2A). *IgH* V(D)J recombination occurs "*stage-specifically*" before that of *IgL* in progenitor ("pro") B cells and is "*ordered*", with D to J_H joining occurring joining occurring before V_H appendage to DJ_H complexes (Cobb et al., 2006). Indeed, V_Hs are not joined to unrearranged D_Hs, even though compatible with 12/23 and B12/23 restrictions. Due to imprecise end-joining, about one in three V(D)J junctions generates an IgH μ chain. Association of a μ chain with a "surrogate" IgL chain forms a "pre-B receptor"(Melchers et al., 2000; Rajewsky, 1996), which gives a "*feedback*" signal to prevent rearrangement of the other allele if in DJ_H configuration (Mostoslavsky et al., 2004) and promote the onset of *IgL* V(D)J recombination at the precursor ("pre") B stage (Cobb et al., 2006). Igκ V(D)J recombination usually occurs before that of Igγ, with most Igγ B cells derived from pre-B cells that, via V(D)J recombination, inactivated both Igκs (Cobb et al., 2006). Association of IgL chains with μ

IgH chains to form non-auto-reactive BCRs down-regulates RAG in newly generated B cells. However, if the BCR is auto-reactive, continued RAG expression promotes “receptor editing” in which the productive V_LJ_L is replaced by another one, potentially generating a non-auto-reactive BCR (Nemazee, 2006).

Higher-order Regulation of V(D)J Recombination

Lineage, stage, order, and feedback regulation of V(D)J recombination are mediated “epigenetically” by modulating “accessibility” of particular loci or regions of loci to RAG (Sleckman and Oltz, 2012). Mouse *IgH* contains several hundred V_Hs, 13 Ds and four J_Hs in an approximately 3-Mb region upstream of the C_Hs (Figure 2B; Figure 3). V(D)J recombinational accessibility and transcription are tightly correlated in *IgH*. In this regard, the J_H to Cμ intronic enhancer (“iEμ”) is required for V_H to DJ_H joining, but not D to J_H joining, which relies on undefined accessibility elements (Subrahmanyam and Sen, 2012). Transcription of unrearranged (“germline”) V_Hs in pro-B cells that is down-regulated in more mature stages was the first accessibility correlate for V(D)J recombination (Sleckman and Oltz, 2012). Widespread antisense transcription also occurs throughout the V_H locus (Matheson and Corcoran, 2012). While it has been debated as to whether transcription directly leads to accessibility (Cobb et al., 2006), these two processes were linked in the TCRα and TCRβ loci (Abarrategui and Krangel, 2007; Cobb et al., 2006). In addition, there are numerous epigenetic accessibility correlates, such as the histone H3 lysine 4 tri-methylation ($H3K4^{me3}$) transcriptional mark enriched around J_Hs in early pro-B cells in association with germline transcription (Subrahmanyam and Sen, 2012). The C-terminal RAG2 plant homeodomain (PHD) binds $H3K4^{me3}$, recruiting RAG2 to transcribed genomic regions with RAG1 binding to RSSs (Desiderio, 2010; Matthews and Oettinger, 2009; Schatz and Swanson, 2011). Such binding specificities accumulate RAG1 and RAG2 within recombination “centers”, where they capture complementary RSSs and initiate V(D)J recombination (Schatz and Ji, 2011; Matthews and Oettinger, 2009).

V_H to DJ_H joining, as opposed to D to J_H joining, is regulated in the context of lineage, order and feedback, which suggested that the V_H to D intergenic region might contain potential regulatory elements (Matheson and Corcoran, 2012). In this regard, targeted mutation of two closely linked CTCF-binding elements (“CBEs”) within the downstream portion of the V_H-D intergenic region led to premature transcriptional activation of D-proximal V_Hs along with associated epigenetic modifications; correspondingly, these CBE mutations also abrogated lineage, stage and feedback control of proximal V_H rearrangement (Guo et al., 2011b; Figure. 2B). Balancing antibody repertoires requires representation of all V_Hs in the vast newly generated pre-B cell population, providing a “primary” repertoire for molding by cellular selection (Melchers et al., 2000; Rajewsky, 1996). While D-proximal V_Hs normally rearrange more frequently than “distal” V_Hs in WT pro-B cells, the most D-proximal V_H (“V_H81X”) greatly predominates on V_H-D intergenic CBE-mutant alleles (Guo et al., 2011b; Figure 2B). Thus, the V_H-D intergenic CBEs help balance primary $V_H(D)J_H$ repertoires by suppressing premature, unordered rearrangement of the most proximal V_Hs; thereby, preventing them from blocking DJ_H substrates for distal V_H rearrangements (Guo et al., 2011b). Consistent with roles in other loci (Phillips and Corces, 2009), these V_H-D intergenic CBE suppressive functions may involve sequestration of the D and J_H portion of

IgH within a distinct regulatory domain at the D to J_H rearrangement stage to insulate proximal V_Hs from premature activation by iEμ or other elements (Degner et al., 2011; Guo et al., 2011b; Figure 2B). Obviously, such insulating activities somehow must be neutralized or circumvented following formation of DJ_Hs to allow joining of V_Hs (Figure 2C).

The mouse *IgH* has a remarkable number and organization of CBEs (Bossen et al., 2012; Degner et al., 2011), which is largely conserved in humans. In addition to two V_H-D intergenic CBEs, there are 10 CBEs ("3'CBEs") clustered just downstream of *IgH*, a CBE adjacent to each proximal V_H RSS, and CBEs interspersed between distal V_H clusters (Figure 2B). Notably, the two V_H-D intergenic CBEs have opposite orientations and the 3'CBEs and V_H CBEs have opposite orientations in both mouse and human (Guo et al., 2011b; Bryne et al., 2008); given possible orientation-specific CBE activity (MacPherson and Sadowski, 2010), this striking organization may contribute to overall CBE function within *IgH*. In this regard, different aspects of *IgH* CBE organization might contribute different functions; for example, suppression versus activation of V_H to DJ_H rearrangement (Bossen et al., 2012; Guo et al., 2011b). In pro-B cells, the intergenic CBE locale forms loops with the iEμ locale, which lacks CBEs, as well as with the 3'CBEs and with some V_H locales (Degner et al., 2011; Guo et al., 2011a; Guo et al., 2011b). Potential roles for such loops in regulation of *IgH* V(D)J recombination has been speculated (Degner et al., 2011; Guo et al., 2011a; Guo et al., 2011b) but not yet directly tested. Indeed, deletion of eight CBEs of the ten 3'CBEs had no obvious impact on IgH V(D)J_H recombination (Volpi et al., 2012); but, the two remaining 3'CBEs might still provide requisite functions.

A more general question is how CTCF binding to CBEs mediates specific regulatory events, given that CTCF binds numerous genomic CBEs and occupies *IgH* CBEs throughout B cell development (Degner et al., 2011). Bound CTCF activities may be influenced by interacting proteins, such as cohesin (Degner et al., 2011), which regulates *TCRα* transcription and recombination (Seitan et al., 2011). In addition, bound CTCF activity could be modified by other factors binding in close proximity. In this regard, the two V_H-D intergenic CBEs have been speculated to belong to a greater regulatory region termed intergenic control region-1 (IGCR1), that also contains binding sites for other factors including Pax5 and YY1 (Guo et al., 2011b), which implicated in control of *IgH* V(D)J recombination and/or formation of various loops within *IgH* that could influence V(D)J recombination (Degner et al., 2011; Ebert et al., 2011; Guo et al., 2011a; see below). Potential roles for these and other factors in mediating IGCR1 CBE-specific or other regulatory events should be clarified by mutation of these binding sites within IGCR1 or in other *IgH* regions. Finally, comparison of the effects of deleting the entire V_H-D intergenic region (Giallourakis et al., 2010) with those of deleting just IGCR1 suggests that the V_H-D interval contains additional regulatory elements that influence generation of long anti-sense transcripts initiated in the downstream D_H cluster (See Supp. Discussion in Guo et al., 2011b).

Balancing primary V_H repertoires relies majorly on large-scale "repositioning" of the distal V_Hs into close physical proximity with the DJ_Hs (Figure 2C). This "locus contraction", which involves formation of chromosomal loops or rosettes (Bossen et al., 2012; Hewitt et al., 2010), is critical for distal V_H utilization and requires several transcription factors including Pax5, YY1, and Ezh-2, (Hewitt et al., 2010; Medvedovic et al., 2011). As distal

V_Hs in "de-contracted" *IgH* loci in Pax5-deficient pro-B cells remain robustly transcribed, locus contraction provides accessibility via physical juxtaposition, as opposed to more standard chromatin-mediated forms of epigenetic accessibility (Hewitt et al., 2010; Medvedovic et al., 2011; Fig 2C). Locus contraction does not require the V_H-D intergenic CBEs (Guo et al., 2011b); although these elements still may promote more local interactions between V_Hs and DJ_Hs of contracted loci that contribute to their synapsis in recombination centers (Bossen et al., 2012; Guo et al., 2011a; Guo et al., 2011b). Candidates for upstream elements that contribute to *IgH* locus contraction are the Pax5-activated intergenic region ("PAIR") elements, which contain CBEs, promoters and Pax5 binding sites; and which are interspersed between upstream V_H clusters (Ebert et al., 2011). A question of interest is whether locus contraction and de-contraction are active mechanisms that occur rapidly in G1-arrested pro-B cells, or whether such genome organizational changes require cell-cycle progression. Active movement would potentially have great relevance for chromosomal translocation mechanisms (see below). Finally, after contraction, more local movements of V_Hs in and out of V(D)J recombination centers theoretically could contribute to balancing their utilization (Lucas et al., 2011; Figure 2C).

Allelic exclusion of Ig gene expression was discovered based on allotypic markers that distinguish surface expression of two Ig alleles (Mostoslavsky et al., 2004). Alleic exclusion results from productive rearrangement of a single *IgH* and a single *IgL* allele that contribute to the BCR of each newly generated B cell. Allelic exclusion may be required to allow cellular selection mechanisms to efficiently discriminate between cells with self-reactive and non-self-reactive specificities. However, precise physiologic roles are enigmatic, because allelic exclusion has not been deregulated at a broad level. While premature V_H81X rearrangements on IGCR1-deleted alleles escape feedback regulation, their expression largely is excluded by cellular selection (Guo et al., 2011b), due to poor pairing of V_H81X containing IgH chains with surrogate IgL chains and their frequent contribution to auto-reactive BCRs (Melchers et al., 2000). Distal V_Hs do not rearrange frequently enough in IGCR1 mutants to test for allelic exclusion. However, locus "de-contraction" mechanisms likely prevent rearrangement of more distal V_Hs by spatially isolating them from DJ_Hs upon formation of productive $V_H(D)J_H$ alleles (Hewitt et al., 2010). If so, cis-elements that regulate *IgH* locus contraction/de-contraction must be defined and, if possible, neutralized to elucidate allelic exclusion roles in normal physiology.

SHM and IgH Class Switch Recombination

Programmed Genomic Alterations in Mature B Cells

AID-dependent CSR and SHM occur in response to antigen-activation of B cells in peripheral lymphoid organs such as spleen and lymph nodes. SHM diversifies primary antibody repertoires by introducing high-frequency point mutations into *IgH* and *IgL* variable region exons (Di Noia and Neuberger, 2007; Figure 3A,B) and occurs in specialized germinal centers (GCs) where B cells are selected for SHMs that generate BCRs with increased antigen affinity (Victora and Nussenzweig, 2012). SHM occurs along V(D)J exons, but is focused around 3–4 nucleotide-long SHM motifs (Di Noia and Neuberger, 2007). Within V(D)J exons, SHM motifs are most abundant in three regions that encode

antigen contact portions of IgH and IgL chains, yielding the most impact of SHM on antigen binding (Di Noia and Neuberger, 2007). CSR takes place inside and outside GCs and replaces Cμ with other C_Hs (e.g., Cγ, Cε, Cα) from the 200-kb region downstream (Figure 3A, C) effecting class switching from IgM to other IgH isotypes (e.g., IgG1, IgE, IgA, etc.) (Chaudhuri et al., 2007). Long (1–10 kb) repetitive switch (S) regions, rich in SHM motifs, precede each C_H. AID-dependent DSBs in the donor S region upstream of Cμ (Sμ) and a downstream acceptor S region initiate CSR in the G1 phase (Nussenzweig and Nussenzweig, 2010) with end joining fusing the two broken S regions to juxtapose the downstream C_H and the V(D)J exon (Figure 3C). CSR is depicted with inherent "deletional" directionality (e.g., Chaudhuri et al., 2007; Figure 3C). However, in contrast to V(D)J recombination, which has an inherent directionality provided by requisite coding and RSS joins, if and how CSR directional joining is imposed are intriguing questions. Finally, CSR is not allelically excluded and usually occurs on both *IgH* alleles (Chaudhuri et al., 2007).

Different Outcomes of AID Activity during SHM and CSR

AID is a small (24 kDa) single-strand (ss) DNA-specific cytidine deaminase that initiates SHM and CSR (Di Noia and Neuberger, 2007). In this context, AID-generated cytidine deamination lesions are processed, at least in part, by general cellular base excision and/or mismatch repair factors to yield mutations in V(D)J exons during SHM and DSBs (plus mutations) in S regions during CSR (Di Noia and Neuberger, 2007; Figure 3B, C). How normal repair activities are diverted into generating mutations and DSBs is of great interest. Possibilities include influences from the nature and/or density of initiating lesions, as well as potential expression of unknown factors that influence normal repair outcome. A related question is how AID activity causes mutations during SHM versus DSBs during CSR (Figure 3B, C). One likely factor is target sequence, with S regions having a higher SHM motif density and being rich in the AGCT sequence, a palindromic SHM motif that targets AID on both DNA strands (Han et al., 2011; Xu et al., 2012). More isolated AID lesions in V regions may be channeled into mutations; whereas, more frequent and dense AID-initiated lesions on S regions might result in opposing nicks or gaps leading to DSBs. As SHM and CSR can occur independently, differential recruitment of downstream factors that influence repair outcome is another possibility.

Factors that Target AID Activity during SHM and CSR

Factors that bring AID to specific targets are of great interest given its potent mutagenic activity and expression in non-lymphoid cells, including germ cells where it has been linked to reprogramming (Fritz and Papavasiliou, 2010). Transcription plays a major role in AID targeting. With respect to CSR, transcription targets AID to particular sets of S regions, thereby, directing CSR (Chaudhuri et al., 2007; Figure 3A, C). In this regard, each C_H has a transcriptional promoter and a non-coding exon ("I-region") upstream of the S region. Through cell surface receptor interactions and cytokine secretion, T cells and other immune cells activate B cells to turn on particular I-region promoters, with resulting "germline" transcripts running through associated S regions to provide AID access (Figure 3A,C; Chaudhuri et al., 2007). In this way, the immune system directs CSR to generate antibody classes most suitable for pathogen elimination. The 3'IgH regulatory region (3'IgHRR) downstream of *IgH* regulates I-region promoters and, thereby, AID targeting over 100-kb

distances (Pinaud et al., 2011). Transcriptional pausing has been implicated in AID targeting (Pavri and Nussenzweig, 2011). In this regard, AID association with Spt5, a transcription cofactor activated by RNA polymerase II pausing, contributes to AID recruitment (Pavri et al., 2010; Figure 3D).

AID targets both non-template and template strands of transcribed duplex DNA during SHM and CSR (Liu and Schatz, 2009). Therefore, once AID arrives, both DNA strands of duplex DNA targets must be available as ssDNA substrates. Negative supercoils or other transcriptionally generated structures might contribute (Longerich et al., 2006). In the latter context, transcription of mammalian S regions generates ssDNA within R-loops, providing AID access to the non-template strand (Chaudhuri et al., 2007; Yu and Lieber, 2003). For variable region exons or frog AGCAGCT-rich S regions that do not favor R-loop generation, transcription-dependent non-template strand access can be promoted *in vitro* by phosphorylation-dependent AID association with replication protein A (RPA), an ssDNA-binding complex (Chaudhuri et al., 2007; Pavri and Nussenzweig, 2011). Finally, the RNA exosome, a large RNA degradation complex, provides AID access to both strands of transcribed AID substrates *in vitro*, theoretically via displacement of nascent transcripts (Basu et al., 2011; Figure 3D). Additional AID-interacting factors, potentially implicated in AID targeting, have been discussed in depth (Xu et al., 2012).

Answers to questions of how transcription, target sequences and/or co-factors differentially and relatively specifically target AID will enhance understanding of how collateral damage is minimized and how highly-specific immune responses are generated. Specific direction of AID activity to different closely linked targets within the *IgH* locus is strikingly illustrated by differential AID-targeting into S regions, but not into adjacent $V_H(D)J_H$ exons during CSR; and by robust SHM of $V_H(D)J_H$ exons in some GC B cells that have not undergone CSR (Liu and Schatz, 2009; Figure 3B,C). How is such specificity achieved? By analogy to transcriptional targeting of one versus another closely linked S region, some aspect of differential transcription of S regions versus V(D)J exons in the two settings could be involved. However, as many other genes are transcribed in B cells undergoing SHM and CSR, some of which do provide lower level sites of “off-target” AID activity (see below), other factors including structural aspects of target sequences and/or specific co-factors may also play a role. In this regard, elucidating factors that target AID to V(D)J exons in the GC response may contribute to designing approaches to elicit certain therapeutic antibodies, such as broadly neutralizing anti-HIV antibodies that are associated with extensive SHM (Wu et al., 2011).

DSB End-Joining during V(D)J Recombination and CSR

Classical Non-Homologous End-Joining

The strict dependence of V(D)J recombination on C-NHEJ facilitated discovery of this repair pathway (Boboila et al., 2012). In mammalian cells, C-NHEJ and homologous recombination (HR) are major DSB repair pathways. HR functions in post-replication repair and requires large stretches of homology (Bassing and Alt, 2004; Heyer et al., 2010); whereas C-NHEJ repairs DSBs throughout the cell cycle, utilizing a spectrum of ends ranging from those lacking homology (“direct joining”) to those employing short “micro”-

homologies (“MHs”) (Boboila et al., 2012). C-NHEJ is critical for DNA repair in G1-arrested somatic cells in which HR does not function. DSB repair by end-joining requires DSB recognition, tethering, processing if needed, and ligation. For C-NHEJ, the Ku70 and Ku80 end-binding complex (“Ku”) recognizes DSBs and the XRCC4 and Ligase 4 (Lig 4) complex joins them (Boboila et al., 2012; Figure 1). C-NHEJ of all types of broken DNA ends depends on these “core” activities. In addition, Ku recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) that activates end processing by the Artemis endonuclease (Lieber, 2010; Soulas-Spraeuel et al. 2007; Figure 1). Several DNA polymerases may contribute to end polishing (Lieber, 2010).

C-NHEJ contributes tremendously to diversifying primary antibody and TCR repertoires by creating diversity in V(D)J junctions, which encode antigen contact regions (Davis and Bjoerkman, 1988; Bassing et al., 2002). RAG contributes by generating covalently sealed (“hairpin”) coding ends and blunt 5'-phosphorylated RSS ends (Figure 1). As RAG-cleaved RSS ends are perfect C-NHEJ substrates, they usually are directly ligated with no additions or losses. However, hairpin coding ends must be opened by Artemis, which cleaves at or near the apex, contributing to deletions and short palindromic insertions (Lieber, 2010). Terminal deoxynucleotidyl transferase (TdT), a lymphocyte-specific V(D)J recombination component, further diversifies repertories by adding non-templated nucleotide additions, termed “N regions”, to coding junctions (Bassing et al., 2002; Figure 1). Junctional diversification mechanisms increase primary antibody and TCR diversity by many orders of magnitude (Davis and Bjorkman, 1988). Restriction of V(D)J joining to C-NHEJ by RAG also may exclude MH-biased A-EJ pathways that could restrict diversity. In this regard, certain fetal Ig and TCR repertoires generated in the absence of TdT have restricted diversity associated with lack of N regions and repeated “canonical” V(D)J junctions promoted by larger than normal MHs (Gilfillan et al., 1995; Bassing et al., 2002).

Alternative End-Joining

MH-biased A-EJ was discovered via linear plasmid re-joining assays in C-NHEJ deficient yeast and mammalian cells and later found to function in CSR in the absence of C-NHEJ (Boboila et. al., 2012). MH is not an absolute requirement for nor absolute signature of A-EJ (Boboila et al., 2012; Lieber, 2010). Factors thus far implicated in A-EJ (including XRCC1, Lig3, PARP1, MRE11, CtIP) each have critical roles in other DNA repair pathways (Boboila et al., 2012). Thus, a suggested working definition of A-EJ, which may reflect several pathways, is end joining in the absence of any of the core C-NHEJ factors (Zhang et al., 2010). Strongly MH-dependent A-EJ in the absence of XRCC4 or Lig4 likely utilizes Ku and other upstream C-NHEJ components with a different ligase (Boboila et al., 2010b; Lieber, 2010). However, B cells lacking Ku or both Ku and Lig4 also undergo CSR at 20–40% WT levels and generate frequent chromosomal translocations by an A-EJ pathway distinct from C-NHEJ, as it requires neither recognition nor joining components of C-NHEJ (Boboila et al., 2010b). As CSR in the absence of Ku plus Lig4 employs more direct joins than CSR in the absence of Lig4 alone, these two A-EJ pathways may be different (Boboila et al. 2010b). In this regard, the embryonic lethality of Lig4-deficient mice, which is associated with severe neuronal apotosis, is rescued by Ku deficiency (Frappart and

McKinnon, 2008); potentially due to increased access of neuronal DSBs to an A-EJ pathway suppressed by Ku bound to DSBs.

The ATM-dependent DSB Response

DSBs, including those involved in V(D)J recombination activate the ATM-dependent DNA damage response (DDR) in which ATM phosphorylates numerous substrates that mediate cell-cycle checkpoints and DNA repair (Nussenzweig and Nussenzweig, 2010). ATM-phosphorylated p53 mediates the G1/S checkpoint that arrests or eliminates cells with unrepaired DSBs (Bassing and Alt, 2004). ATM also phosphorylates a set of chromatin-associated proteins, including histone H2AX and 53BP1, that form foci over hundreds of kilobases flanking DSBs (Nussenzweig and Nussenzweig, 2010). Beyond contributing to checkpoints, such foci may contribute directly to DSB joining, for example by end tethering (Bassing and Alt, 2004). ATM has a clear role in V(D)J recombination, as ATM deficiency leads to premature release of some V(D)J coding ends from post-cleavage RAG complexes and to moderately impaired lymphocyte development (Helmink and Sleckman, 2012). On the other hand, deficiency for 53BP1 has only a very modest impact, and deficiency for H2AX no detectable impact on lymphocyte development or V(D)J recombination (Helmink and Sleckman, 2012). However, all of these factors actually can serve more major roles in C-NHEJ during V(D)J recombination that are masked by functional redundancies with XRCC4-like factor (XLF) (Helmink and Sleckman, 2012; Figure 1).

XLF, which interacts with XRCC4, is mutated in radiosensitive, immunodeficient patients (Lieber, 2010; Soulas-Sprauel et al., 2007). In this regard, XLF deficiency is associated with lymphopenia in humans and leads to impaired V(D)J recombination within extra-chromosomal substrates in human fibroblast lines and mouse embryonic stem cells and embyronic fibroblasts that ectopically express RAG (Boboila et al., 2012; Soulas-Sprauel et al., 2007). Yet, XLF deficiency in mice does not markedly impact V(D)J recombination in developing lymphocytes or pro-B cell lines, which suggested the potential existence of compensatory factors (Li et al., 2008). Correspondingly, XLF was found to be functionally redundant with the broader ATM-dependent DDR for chromosomal V(D)J recombination and C-NHEJ (Boboila et al., 2012). Thus, mice with combined deficiencies for XLF and ATM or XLF and 53BP1 have severe impairments of V(D)J recombination, lymphocyte development, and C-NHEJ (Zha et al., 2011; Liu et al., 2012; Oksenych et al., 2012). While combined XLF and H2AX deficiency is lethal, conditional inactivation revealed substantial XLF and H2AX functional redundancy in V(D)J recombination (Zha et al., 2011). The basis for the functional redundancy between XLF and the ATM-dependent DDR in chromosomal V(D)J recombination and C-NHEJ is unknown. One general possibility is redundant functions, for example in end tethering (Zha et al., 2011). Another possibility is different functions that are compensatory; for example, the DDR may tether DSBs allowing more time for repair; while XLF may enhance C-NHEJ recruitment, allowing repair before DSBs separate (Zha et al., 2011).

End-Joining of S-region DSBs during CSR

Joining of many S-region DSBs during CSR employs C-NHEJ (Boboila et al., 2012). In the absence of C-NHEJ, CSR is reduced to 20–40% of normal and many AID-initiated S-region

DSBs remain un-joined and progress to chromosome breaks or translocations. As outlined above, residual CSR in C-NHEJ-deficient B cells is mediated by A-EJ (Boboila et al., 2012). Thus, unlike RAGs in V(D)J recombination, AID does not chaperone CSR joining specifically into C-NHEJ. In this context, CSR DSB joining likely reflects general chromosomal DSB repair via end joining (Zarrin et al., 2007). Deficiency for ATM or H2AX alone impairs CSR more than V(D)J recombination, with levels reduced to 50% of normal (Nussenzweig and Nussenzweig, 2010). Combined deficiency for ATM and XLF further reduces CSR to levels observed in C-NHEJ-deficient cells with predominantly MH-mediated junctions, suggesting ATM and XLF have functional redundancy for C-NHEJ but not A-EJ (Zha et al., 2011). Notably, 53BP1 deficiency nearly abrogates CSR, suggesting specialized 53BP1 CSR roles beyond any served in the general DDR. Implicated roles include promoting synapsis of two broken S-region ends, protecting them from resection and/or guiding choice of end-joining pathway (Bothmer et al., 2010; Bothmer et al., 2011; Boboila et al., 2012). Potential 53BP1 CSR roles prior to DSB formation have not been ruled out. Deficiencies for ATM, H2AX, or 53BP1 in B cells activated for CSR lead to high levels of AID-dependent *IgH* chromosome breaks and translocations, similar to C-NHEJ deficiencies; yet, there is much less general genomic instability associated with 53BP1 deficiency compared to that observed in the context of other DDR deficiencies or C-NHEJ deficiencies (Boboila et al., 2012), potentially suggesting functional compensation for 53BP1 deficiency in end-joining outside of CSR.

Mechanisms Promoting CSR versus Intra-S-region DSB Joining

AID-initiated DSBs within targeted S regions occur very frequently. Within a given S region, some DSBs may simply be rejoined; while others are rejoined with large resections or joined to other DSBs in the same S region to generate intra-S deletions (“ISDs”) (Figure 3C). Unlike RAG, AID does not require synapsis of two different target sequences to generate DSBs; raising the question of how the potential intra-S region joining of DSBs is balanced with inter-S region joining to promote physiological CSR joining levels (Zarrin et al., 2007; Boboila et al., 2012). Core C-NHEJ factors and 53BP1 may play a role as, in their absence, S-region DSBs are more frequently joined as ISDs versus CSR junctions (Boboila et al., 2010a; Bothmer et al., 2010). Notably, the yeast I-SceI meganuclease induces recombinational class-switching in activated B cells in which S regions are replaced with I-SceI targets (Zarrin et al., 2007), suggesting DSB synapsis during CSR evolved to exploit general cellular mechanisms, such as the DDR or features of three-dimensional (3D) spatial genome organization (see below). The ability of I-SecI-mediated *IgH* DSBs to join to AID-initiated DSBs 100 kb away in a translocation-like process (Zarrin et al., 2007) provided the basis for development of high-throughput genome-wide “DSB/translocation cloning” approaches that detect genome-wide DSBs via joining to I-SceI- or RAG-generated DSBs in fixed locations (Chiarle et al., 2011; Klein et al., 2011; Helmink et al., 2012).

Mechanisms of Chromosomal Translocations in Lymphocytes

Roles of Oncogenic Selection and Mechanistic Factors in Recurrent Translocations

Recurrent oncogenic translocations occur in tumor progenitors as rare events that are highly selected for impact on cellular proliferation or survival. Activation of oncogenes

translocated into *Ig* or *TCR* loci involves their dysregulation by bringing them under the influence of strong antigen receptor locus enhancers (Janz, 2006). Such enhancer activity has been proven for the 3'IgHRR; which, in a mouse lymphoma model, activates *c-myc* over long distances subsequent to translocation into *IgH*(Gostissa et al., 2011). The 3'IgHRR likely serves the same role in many human tumors with oncogenic *IgH* translocations (Janz et al., 2006). However, basic mechanistic factors also can contribute substantially to translocation frequency of given sequences and, thereby, even influence which oncogenes are translocated in particular tumors (Gostissa et al., 2009; Wang et al., 2009). At a gross cytogenetic level, chromosomal translocations are defined as exchanges between different non-homologous chromosomes. Cancer genome analyses revealed that many translocations result from end joining of two separate DSBs (Stratton et al., 2009). Therefore, at a molecular level, genome rearrangements that join two separate DSBs on the same chromosome (e.g., as in CSR) could be considered translocations. Within a cell population, several mechanistic variables influence translocation frequency between two genomic sites (Zhang et al., 2010; Zhang et al., 2012; Figure 4A). A major variable is the frequency of DSBs at each site available for joining, which itself would be influenced by rate of DSB initiation and the time DSBs persist. Another variable is the frequency with which the two sites are physically juxtaposed (“synapsed”). Finally, physically proximal sequences that harbor DSBs simultaneously must be fused to form a translocation, potentially providing additional variables related to joining pathway access.

Sources of Translocation-prone DSBs Involved in Ig and TCR Translocations

RAG- or AID-initiated DSBs introduced into antigen receptor loci during attempted V(D)J recombination or CSR, respectively, have been implicated in oncogenic translocations found in many human lymphoid cancers (Gostissa et al., 2011; Robbiani and Nussenzweig, 2012). Ablation of RAG or AID in mouse models confirmed such AID and RAG roles (Gostissa et al., 2011; Robbiani and Nussenzweig, 2012). AID also may generate translocation-initiating DSBs in *IgL* variable region exons during SHM (Robbiani and Nussenzweig, 2012). Many RAG-initiated antigen receptor locus DSBs involved in translocations likely are initiated during the normal V(D)J recombination process within a given Ig or TCR locus (Gostissa et al., 2011). In this regard, inter-chromosomal V(D)J recombination with cryptic RSSs elsewhere in the genome is likely dampened by 12/23 or B12/23 restrictions and by constraints imposed by genome organization (see below). However, a role for inter-chromosomal V(D)J recombination has been implicated in the genesis of some oncogenic translocations, such as those found in certain T-ALLs (Gostissa et al., 2011; Nambiar et al., 2008).

DSBs that serve as partners for translocation of antigen receptor locus DSBs, among other sources, could come from off-target RAG or AID activity. Even though RAG2 binds widely across the genome (Ji et al., 2010), involvement of RAG in DSBs outside of antigen receptor loci would likely be subject to the same restrictions that limit inter-chromosomal V(D)J recombination. However, biochemical studies showed that RAG nicks cryptic RSSs and non-B DNA structures (Tsai and Lieber, 2010; Schatz and Swanson, 2011). In this regard, analyses of oncogene translocation hotspots in cancers that arise from developing human B cells led to the proposal that AID expression in early B cells targets CpG sites within certain

zones resulting in generation of substrates for RAG- and Artemis-dependent nicking and, ultimately, in generation of DSBs (Tsai and Lieber, 2010; Cui et al., 2012). There is ample evidence that off-target AID activity leads to DSBs involved in translocations. AID mutates numerous genes, beyond Ig genes, in GC B cells and B cells activated *in vitro* for CSR (Liu and Schatz, 2009; Pavri and Nussenzweig, 2011). Moreover, in activated B cells, DSB/translocation cloning showed that AID generates off-target DSBs in various genes, including multiple known B-cell oncogenes, making them the major endogenous source of translocation hotspots in these cells (Chiarle et al., 2011; Klein et al., 2011). Going forward, an important goal will be to elucidate off-target AID DSBs and other sources of DSBs in GC B cells, from which many human lymphomas arise.

Translocation partner DSBs for RAG- or AID-initiated DSBs may be generated by cell-intrinsic mechanisms, including oxidative and replication stresses, and by transcription (Tsai and Lieber, 2010; Kim and Jinks-Robertson, 2012; Barlow et al., 2013). In activated B cells, transcription is associated with both AID-initiated and AID-independent DSBs that are translocation targets (Chiarle et al., 2011; Klein et al., 2011). Transcription may generate DSBs through various mechanisms including collision with replication forks or generation of DSB-prone DNA structures (Kim and Jinks-Robertson, 2012). In the latter context, several DNA sequence-related structures have been implicated in generating DSBs, including R-loops and non-B DNA structures (Zhao et al., 2010; Kim and Jinks-Robertson, 2012). As one *bona fide* example, *IgH* S regions are prone to DSBs and, correspondingly, are translocation hotspots in activated AID-deficient B cells, potentially because they form R-loops or other unstable structures during S-region transcription (Chiarle et al., 2011). Recent studies have shown that regions containing CpG nucleotides also may be particularly susceptible to AID-independent replication fork collapse; these regions, termed “Early Replication Fragile Sites”, are found at certain translocation breakpoints in B cell lymphomas (Barlow et al., 2013). Finally, various cell-extrinsic agents broadly induce DSBs; among these are a number of cancer therapeutics, such as topoisomerase inhibitors and ionizing radiation (IR) (Tsai and Lieber, 2010).

Role of DSB Persistence in Translocations

Deficiencies for C-NHEJ or DDR-responses lead to persistence of RAG- or AID-initiated DSBs in antigen receptor loci and elsewhere that progress into translocations (Zhang et al., 2010). Dangers associated with such persistence are controlled, in many cases, by strict checkpoints that eliminate cells with persisting DSBs, for example, the p53-dependent G1 checkpoint (Helmink and Sleckman, 2012). In the latter context, combined deficiency for C-NHEJ and p53 or H2AX and p53 predisposes to recurrent pro-B lymphomas with RAG-initiated *IgH* to *c-myc* translocations; while deficiency for any one of these factors does not (Gostissa et al., 2011). ATM deficiency, which impacts both V(D)J joining and the p53-dependent G1-checkpoint, predisposes to B and/or T cell lymphomas with V(D)J recombination-associated oncogenic translocations in humans and mice (Helmink and Sleckman., 2012). In RAG-expressing ATM-deficient pro-B cell lines, DSB/translocation cloning hotspots, besides *IgH* and *IgL*, include various TCR loci, demonstrating that persistence allows RAG-initiated DSBs at relatively inaccessible antigen receptor locus RSSs to become recurrent translocation targets (Zhang et al., 2012). In ATM-deficient mice,

RAG-initiated *IgH* DSBs generated at the pro-B stage lead to chromosomal breaks in peripheral B cells that provide sources of V(D)J recombination-associated peripheral B-cell translocations (Callen et al., 2007). Whether such RAG-initiated chromosomal breaks actually persist or, rather, lead to generation of new sets of DSBs via a breakage-fusion-bridge type of mechanism, as observed in the dual absence of C-NHEJ and p53 (Bassing and Alt, 2004; Nussenzweig and Nussenzweig, 2010), remains to be determined. Finally, receptor editing in the periphery may also contribute to translocations (Wang et al., 2009).

End-Joining of DSBs to Form Translocations

C-NHEJ-deficient cells often accumulate substantial levels of pre-replicative chromosome breaks as well as chromosomal translocations (e.g., Yan et al., 2007; Franco et al., 2008; Boboila et al. 2010a), consistent with the major role of C-NHEJ in the G1 phase (Gostissa et al, 2011). In this regard, characterized translocation junctions in C-NHEJ-deficient primary lymphocytes and tumors are generated by end joining as opposed to HR-related mechanisms and, thus, must be catalyzed by A-EJ (Boboila et al., 2012). However, a critical question is whether A-EJ is a “translocation-prone” pathway that can drive translocations in the presence of C-NHEJ or whether its predominance in the absence of C-NHEJ reflects an abundance of persistent DSBs that drive a less efficient DSB joining reaction. In this context, some studies suggest A-EJ is especially prone to generating translocations (Simsek and Jasin, 2010). While a role for C-NHEJ in generating translocation junctions has not been ruled out, C-NHEJ has been suggested to normally suppress translocations via a propensity to join DSBs intra-chromosomally, potentially in association with the DDR and enhanced by 3D spatial proximity effects (Boboila et al., 2012; see below).

Role of Spatial Genome Organization in Guiding Translocations

One model for DSB synapsis prior to translocation posits DSB movement, while another posits synapsis of translocating sequences prior to DSB formation (Misteli and Soutoglou, 2009). Cytogenetic studies correlated “proximity” of two loci in the mammalian interphase nucleus with involvement in translocations (Misteli and Soutoglou, 2009), supporting the pre-existing synapsis model. While large-scale DSB movements occur in yeast (Dion et al., 2012; Mine-Hattab and Rothstein et al., 2012), evidence for mammalian DSB movement is more limited (Misteli and Soutoglou, 2009). Most notably, 53BP1 promotes mobility of uncapped telomeres, which resemble DSBs, and their fusion by end joining (Dimitrova et al., 2008). More locally, DSB mobility via Brownian motion also might contribute to synapsis of proximal DSBs (Misteli and Soutoglou, 2009; Zhang et al., 2012).

Both G1-arrested and cycling human cells display cellular heterogeneity in spatial genome organization, allowing loci that on average are not highly proximal in cell populations to be spatially proximal within some cells (Imakaev et al., 2012). Thus, when considering a cell population, translocation frequency of two DSB sites that do not actively move should reflect products of factors related to available DSB frequency at each site (e.g., [fDSB*a* × fDSB*b*]) and a factor describing their synapsis frequency within the population (f*syn*) (Zhang et al., 2012; Figure 4A). Correspondingly, cellular heterogeneity in spatial genome organization allows frequent DSBs to drive recurrent translocations of loci not considered highly proximal by classical cytogenetic approaches; providing an explanation for how 40

pairs of targeted DSBs on various chromosomes translocate recurrently in G1-arrested pro-B lines (Zhang et al., 2012) and how AID-dependent DSBs translocate to target DSBs at higher frequency than predicted by average proximity (Hakim et al., 2012). This model also may explain inter-chromosomal CSR (Pinaud et al., 2011), which could be driven by frequent, simultaneous S-region DSBs on both *IgH* alleles (Chiarle et al., 2011).

When DSB frequencies at one or both of the potentially translocating loci are not dominant compared to those of other DSBs genome wide, factors that influence pre-existing spatial proximity of two loci can markedly influence potential translocation target landscapes (Figure 4B). Such factors include lying in active versus inactive chromatin compartments, being actively transcribed, being on similar-sized chromosomes, and most notably being on the same chromosome (Mahowald et al., 2009; Chiarle et al., 2011; Hakim et al., 2012; Klein et al., 2011; Zhang et al., 2012). In the latter context, Fluorescence *In Situ* Hybridization (FISH) and Hi-C experiments show mammalian chromosomes have territories in both cycling and G1-arrested cell populations, such that intra-chromosomal interactions occur far more frequently than inter-chromosomal interactions (Imakaev et al., 2012; Marti-Renom and Mirny, 2011; Zhang et al., 2012). Correspondingly, in G1-arrested, ATM-deficient pro-B lines treated with IR to “normalize” DSBs genome wide, this aspect of intra-chromosomal spatial genome organization causes regions along the entire length of an individual chromosome to become “hotspots”, for translocations of DSBs introduced *in cis* into that chromosome (Zhang et al., 2012; Figure 4B). Likewise, translocations in tumor cells subsequent to DSB-inducing chemotherapies or radiotherapies might be similarly influenced. The propensity of two DSBs to join intra-chromosomally also might lead to recurrent intra-chromosomal deletions associated with T-ALLs and other cancers, if involved regions are structurally or otherwise prone to DSBs (O'Neil and Look, 2007; Solimini et al., 2012) and may contribute, as well, to extensive intra-chromosomal cancer genome rearrangements known as chromothripsis (Maher and Wilson, 2012).

DSB/translocation cloning analyses of inversional translocation junctions, in G1-arrested, ATM-deficient pro-B lines, in which DSBs are normalized across the genome via IR, revealed that intra-chromosomal translocation frequencies are further elevated in several hundred kilobase-regions flanking DSBs (Zhang et al., 2012; Figure 4B). Given that two random DSBs would rarely occur within such short genomic distances, this phenomenon would most greatly impact joining of closely linked recurrent DSBs, such as AID-initiated S-region DSBs during CSR (Zarrin et al., 2007). While additional studies in wild-type and various other mutant backgrounds should help resolve potential mechanisms underlying this phenomenon, there are several general possibilities, not mutually exclusive, that can be considered. One is that perturbations of local chromatin structure around a DSB or activation and recruitment of repair machinery in association with a DSB might contribute to enhanced joining with proximal DSBs (Bassing et al., 2004; Downs et al., 2007; Zarrin et al., 2007). Another is that such “shorter-range” enhancement of intra-chromosomal joining of separate DSBs may reflect pre-existing spatial proximity influences at a more local level. In this regard, Hi-C analyses indicate that contacts between loci within a 1-Mb distance are more frequent than other intra-chromosomal interactions, suggesting chromatin is more organized at shorter distances and that contacts over longer distances occur stochastically in

only a subset of cells (Fudenberg and Mirny, 2012), consistent with mammalian chromosomes being organized into megabase-sized “topological domains” (Dixon et al., 2012). Such local influences of pre-existing genome organization at the megabase, or potentially sub-megabase, scale also might contribute to synapsis of V_Hs and the DJ_H prior to RAG cutting (Lucas et al., 2011), and conceivably provide a mechanistic basis for highly preferential proximal V_H rearrangement.

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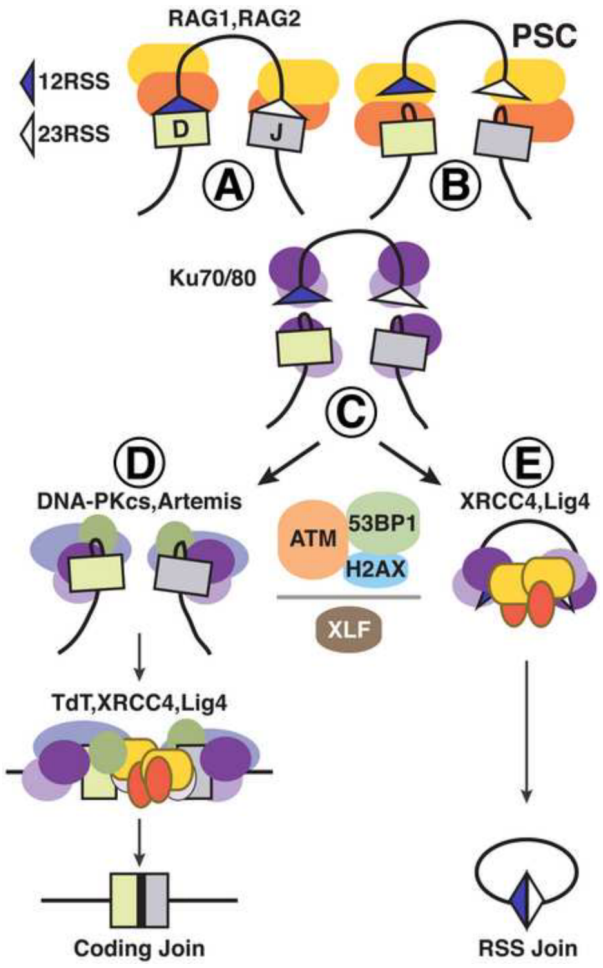

Figure 1. Repair of RAG-induced Antigen Receptor Locus DSBs by Classical Non-Homologous End Joining

(A) RAG1 and −2 (yellow and orange ovals) are targeted to participating gene segments in the context of the 12/23 rule. Triangles represent 12-RSSs (blue) and 23RSSs (white) and boxes represent potential coding segments. (B) RAG holds cleaved hairpin coding and blunt RSS ends in a post-cleavage synaptic complex (PSC) and (C) directs the reaction into C-NHEJ initiated by Ku70 and Ku80 (dark and light purple ovals) binding. (D) Coding ends require processing and N regions can be added by TdT (grey oval) whereas (E) RSS ends are directly ligated by the XRCC4 (yellow oval)/Lig4 (red oval) complex to form coding and RSS joins, respectively. Functional redundancy of DDR and XLF in this reaction is indicated by ATM, 53BP1 and H2AX ovals separated by a line from an XLF oval. Specifically, coding joins are modestly impaired in the absence of the ATM and 53BP1 DDR factors and normal in the absence of the H2AX DDR factor or XLF C-NHEJ factor. However, coding joins are severely impaired in the combined absence of XLF and any one of the three DDR factors. RSS joins are normal in the absence of any one of the DDR factors or XLF but severely impaired in the absence of ATM or 53BP1 (H2AX was not tested) and XLF. See text for details.

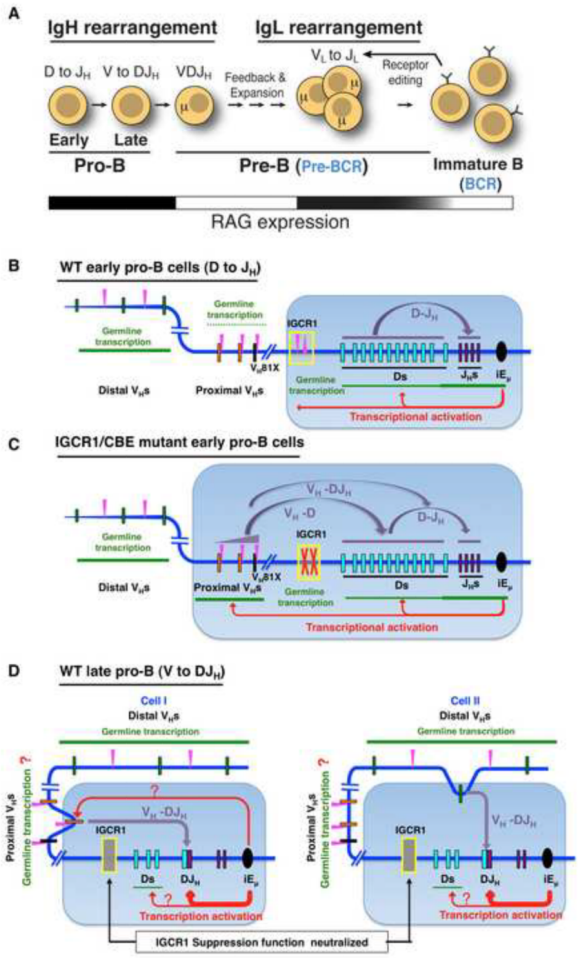

Figure 2. Regulation of V(D)J Recombination during B Cell Development

A) B cell development is directed by ordered assembly of *IgH* and *IgL* genes coupled with feedback mechanisms linking *IgH* and *IgL* expression to developmental progression (see text for details). B) During the D to J_H rearrangement stage in early pro-B cells, prevention of premature, un-ordered proximal V_H rearrangements to germline Ds requires the two IGCR1 CBEs, which may functionally segregate the D and J_H portion of the locus (blue rectangle). Gene segments and elements are indicated with the most proximal V_H segment (V_H81X) shown as a black rectangle. CBEs are indicated as extended pink arrowheads with vertical directi indicating relative sequence orientation. Known, robust germline V_H transcription is indicated by thick green line. while very low level germline V_H transcription is indicated by a thin dotted green line. Known or hypothesized iEμ-mediated transcriptional activation is indicated by red arrows. C) In early pro-B cells in whch the IGCR1 CBEs are inactivated (red crosses), the functional segregation of the D and J_H portion of the *IgH* locus extends to the proximal V_Hs, deregulating their transcription and rearrangement, especially V_H81X. Distal V_Hs are physically unavailable for recombination in these cells due to lack of locus contraction. D) In the late pro-B stage subsequent to DJ_H rearrangement, IGCR1 CBE activities must be neutralized (grey box) to allow V_H segments to enter into V_H to DJ_H "recombination centers" (also indicated by blue rectangles). *IgH* locus contraction occurs at this stage to bring distal V_Hs closer to already recombined DJ_Hs. At this stage both proximal

(left) and distal (right) V_Hs may enter into a recombination center, drawn in this case as occurring in two individual cells although this process has not yet been dissected at this level. Red question marks indicate other speculated aspects of the process that have not been tested experimentally. See text for more details and references.

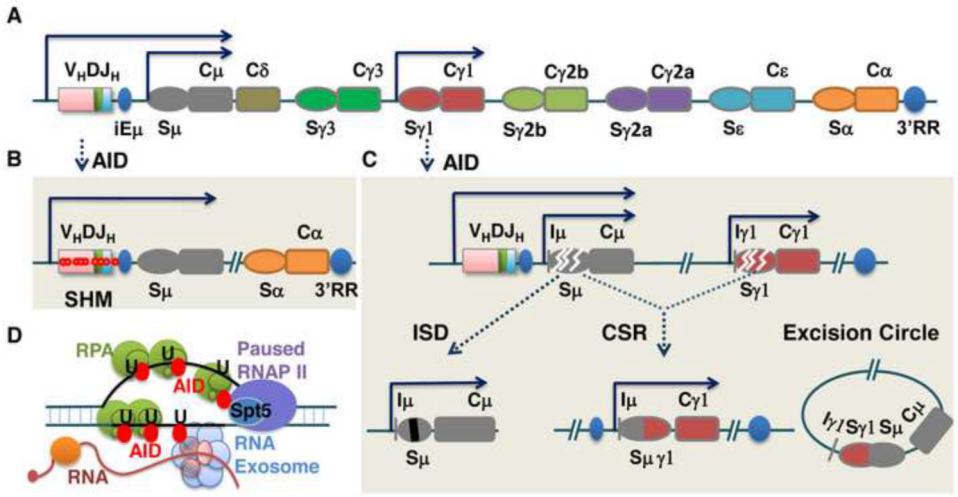

Figure 3. AID Targeting during SHM and IgH CSR

A) Organization of an expressed *IgH* locus with general location of V(D)J exon, iEμ, 3'IgHRR (3'RR) and C_Hs, S regions and I-region promoters (line/arrow). B) During SHM in GC B cells, AID targets transcribed IgH V(D)J exons leading to mutations (red circles) but does not necessarily target downstream S regions. C) During CSR, in activated B cells in culture, AID targets transcribed S regions leading to somatic mutations and DSBs (jagged gaps) but not adjacent V(D)J exons. AID-initiated DSBs in S regions can be joined to form intra-S deletions (ISDs) (truncated S region oval with black center) or CSR events (fused S region ovals). CSR is thought to occur by a deletional mechanism with intervening sequences deleted on an excision circle. D) Working model for transcriptional AID targeting (adapted from Basu et al., 2011). “U” refers to deaminated cytidines; RPA to replication protein A) and RNAP II to RNA Polymerase. See text for other details.

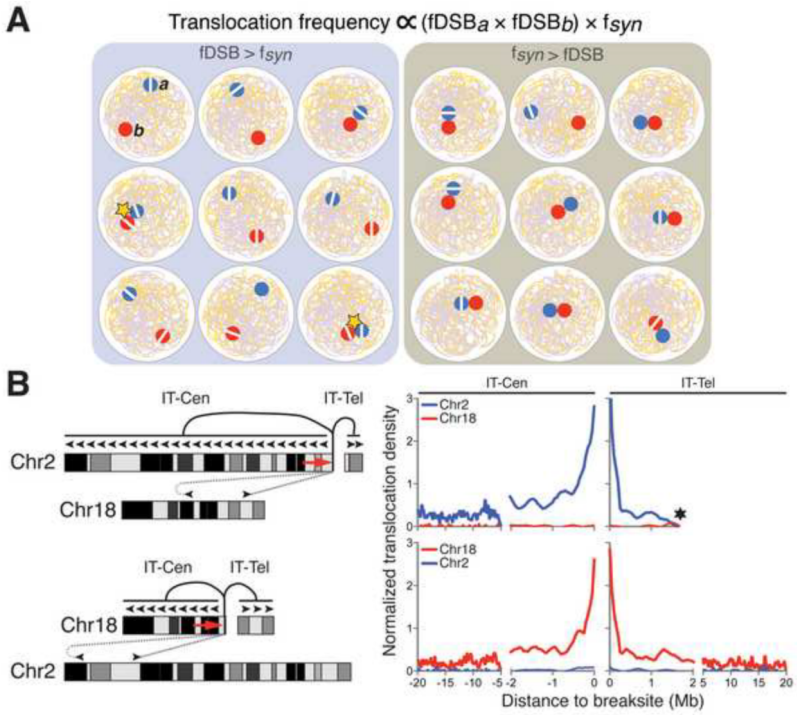

Figure 4. Pre-existing Spatial Organization of the Genome Influences Translocations

A) Cellular heterogeneity of spatial genome organization allows frequent DSBs to drive recurrent translocations. Two genomic loci (*a*, *b*) on heterologous chromosomes are illustrated as red and blue circles; white lines within them indicate DSBs; stars indicate translocations. fDSB indicates frequency of available DSBs at a given site; fsyn represents frequency of cells in the population in which two DSBs are physically juxtaposed. Comparison of left and right panels illustrates that frequent DSBs can drive recurrent translocations of two sequences that are, on average, not the most proximal in a population. B) Pre-existing spatial proximity markedly influences translocation frequency in the absence of dominant DSBs. *Left*, schematic of two experimental examples (Zhang et al., 2012) illustrates that a targeted DSB (gap after red arrowhead) in a given chromosome translocates much more frequently along the same chromosome than to other chromosomes. *Right*, translocation frequencies from a DSB on a given chromosome in ATM-deficient, IR-treated, and G1-arrested transformed pro-B lines are highest to sequences very proximal to the DSB. The curves are based on actual data (from Zhang et al., 2012), shown in schematic form on the left panel, and represent inversional *cis* translocations from the DSB site, with primer orientation indicated by red arrowhead on chromosome ideogram. Inversional translocations (IT) clearly involve joining of two DSBs in this assay and, thus, are distinguishable from resections that also contribute substantially to breaksite-proximal junctions (Chiarle et al., 2012). Black * indicates end of Chr.2. Mb, megabases. Cen and Tel refer to “centromeric” and “telomeric” relative to the DSB. See text for more details.