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V(D)J recombination, somatic hypermutation and class switch recombination of immunoglobulins: mechanism and regulation

Xiying Chi,[1,2*] Yue Li[1,2*] and Xiaoyan Qiu[1,2]

[1]*Department of Immunology, School of Basic Medical Sciences, Peking University, Beijing, and* [2]*NHC Key Laboratory of Medical Immunology, Peking University, Beijing, China*

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*These authors contributed equally to this work.
Correspondence: Xiaoyan Qiu, Department of Immunology, School of Basic Medical Sciences, Peking University, Beijing 100191, China. Email: qiuxy@bjmu.edu.cn
Senior author: Xiaoyan Qiu

Summary

Immunoglobulins emerging from B lymphocytes and capable of recognizing almost all kinds of antigens owing to the extreme diversity of their antigen-binding portions, known as variable (V) regions, play an important role in immune responses. The exons encoding the V regions are known as V (variable), D (diversity), or J (joining) genes. V, D, J segments exist as multiple copy arrays on the chromosome. The recombination of the V(D)J gene is the key mechanism to produce antibody diversity. The recombinational process, including randomly choosing a pair of V, D, J segments, introducing double-strand breaks adjacent to each segment, deleting (or inverting in some cases) the intervening DNA and ligating the segments together, is defined as V(D)J recombination, which contributes to surprising immunoglobulin diversity in vertebrate immune systems. To enhance both the ability of immunoglobulins to recognize and bind to foreign antigens and the effector capacities of the expressed antibodies, naive B cells will undergo class switching recombination (CSR) and somatic hypermutation (SHM). However, the genetics mechanisms of V(D)J recombination, CSR and SHM are not clear. In this review, we summarize the major progress in mechanism studies of immunoglobulin V(D)J gene recombination and CSR as well as SHM, and their regulatory mechanisms.

Keywords: B cell; class switch recombination; mechanism; somatic hypermutation; V(D)J recombination.

Immunoglobulin structure and genetic encoding

Immunoglobulin is comprised of two immunoglobulin heavy (IgH) chains encoded by the IgH heavy-chain locus (chromosome 14 in human and 12 in the mouse) and two immunoglobulin light (IgL) chains encoded by either the Igκ (chromosome 2 in human and 6 in the mouse) or Igλ (chromosome 22 in human and 16 in the mouse) light-chain loci. IgH chains have five major isotypes (Igμ, Igα, Igγ, Igδ and Igε), with four subtypes of IgG (Igγ1, Igγ2,

Abbreviations: 3′RR, 3′ regulatory region; 3′Eα, 3′ Cα enhancer; 53BP1, p53-binding protein 1; AID, activation-induced cytidine deaminase; Alt-NHEJ, alternative NHEJ; APE1/2, apurinic/apyrimidinic endonuclease; ATM, ataxia telangiectasia mutated; Bach2, BTB and CNC homology 2; BER, base excision repair; Blimp-1, B lymphocyte-induced maturation protein-1; BRCA1, breast cancer 1; C-NHEJ, canonic NHEJ; CSR, class switch recombination; CTCF, CCCTC-Binding Factor; DDX1, DEAD-box RNA helicase 1; DNA-PK, DNA protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand breaks; Ebf1, early B-cell factor 1; eEF1A, eukaryotic elongation factor 1 α; Erag, enhancer region of RAG1/2; Exo1, exonuclease 1; FOXO, Forkhead box; G4, G-quadruplexes; HMG, high-mobility group; hs, hypersensitive sites; IgH, immunoglobulin heavy; IgL, immunoglobulin light; Lig, ligase; LSR, locus suicide recombination; Mbd4, methyl-CpG binding domain protein 4; MDC1, mediator of damage checkpoint protein; MLH1, MutL homologue 1; MMR, mismatch repair; NBD, nonamer binding domain; NHEJ, non-homologous end joining; PARP1, poly-ADP ribose polymerase 1; PAXX, paralogue of XRCC4 and XLF; PCNA, proliferating cell nuclear antigen; pol, polymerases; RAP80, receptor-associated protein 80; RPA, replication protein A; RSS, recombination signal sequence; SHM, somatic hypermutation; ssDNA, single-stranded DNA; XLF, XRCC4-like factor; XRCC4, X-ray cross complementing Group 4

Igγ3 and Igγ4) and two subtypes of IgA (Igα1 and Igα2). The IgL chain has two types (Igκ and Igλ). IgH chains have four (Igγ, Igα and Igδ) or five (Igμ and Igε) domains while IgL chains have two domains. The N-terminal regions of heavy and light chains, known as variable (V) region, are highly variable in their sequences and are the antigen-binding portions of the antibody.[1] The C-terminal regions of immunoglobulin, both IgH and IgL, have only a few sequential variations in the same species but different individuals, so are called constant (C) regions.[2] Igγ, Igα and Igδ all have three C_H whereas both Igμ and Igε have four C_H (see Supplementary material, Fig. S1a).[3]

The V region of IgH chain is encoded by V(variable), D(diversity), J(joining) genes whereas the V region of IgL chain is encoded by V and J segments with the absence of D segments in the light-chain loci. The C regions of different immunoglobulin isotypes are encoded by different C_H exon clusters, which are organized in the order of Cμ, Cδ, Cγ, Cε and Cα in the IgH locus.[4] These genes are assembled in the developing lymphocyte by V(D)J recombination to form a complete immunoglobulin (see Supplementary material, Fig. S1b).

V(D)J recombination

The V regions are assembled through V(D)J recombination of V_H, D_H and J_H genes on the heavy chain and V_L and J_L genes on the light chain. This recombination is initiated by double-strand breaks (DSB) produced by the Recombination-activating gene (RAG1–RAG2) recombinase at specific recombination signal sequences (RSS).

Recombination signal sequences

The RSS (see Supplementary material, Fig. S2a) normally consists of a highly conserved heptamer motif (consensus sequence 5′-CACAGTG-3′) and a conserved nonamer sequence (consensus sequence 5′-ACAAAAACC-3′) separated by a poorly conserved spacer sequence of 12 or 23 nucleotides.[3,5] The heptamer sequence is considered to be the essential recognition element. The first three nucleotides of the heptamer (closest to the coding flank) show the highest sequence conservation, and are critical for recombination, whereas the remaining heptamer positions are much less important. The nonamer sequence is dispensable for recombination, with only a few highly conserved positions.

Both the RSS at the 3′ end of the IgH V fragment and the 5′ end of the J fragment carry a 23-bp spacer sequence, while the D fragment has a 12-bp spacer sequence at both the 5′ and 3′ ends of the RSS (see Supplementary material, Fig. S2b).[3] According to the 12/23 rule,[6] efficient recombination only occurs between RSS with different spacer lengths. Therefore, D can interact with J and V while the V cannot be connected to the J because it does not comply with the 12/23 rule. In addition, proximal sites undergo recombination substantially more frequently than distal ones.[6] So the order is first D-J then V-DJ. For the light chain, the 3′ end of the V fragment and the 5′ end of the J fragment are reverse complementarily complementary, with a 12- or 23-bp spacer sequence, respectively, which can result in a V-J linkage.

However, recently, it has been widely proved that the 12/23 rule of genomic recombination is frequently violated under physiological conditions, resulting in unanticipated hybrid recombinations. Non-12/23 junctions under physiological conditions are thought to be rare, the most common being in VDDJ rearrangements of the IgH locus that occur once per 800 cells. In addition to violating the 12/23 rule, other non-canonical rearrangements form hybrid signal-to-coding junctions and are typically generated in artificial systems, but may also be detected at low frequency *in vivo*.[7]

RAG1/RAG2

The V(D)J recombinase consists of two lymphoid-specific proteins, RAG1 and RAG2, of 1040 and 527 residues, which carry out DNA cleavage. Both RAG proteins are large multi-domain proteins that consist of core regions and non-core regions (see Supplementary material, Fig. S3). The catalytic core and DNA-binding functions are largely contained within RAG-1.[6] The 'core' region of RAG1 is required for binding to the nonamer as well as catalysis of cleavage. The non-core domains of the RAG proteins provide important regulatory functions, but the mechanism is not well understood. However, the role for RAG2 is less clear, which may function to activate RAG1 for sequence-specific binding and cleavage, and also provide additional DNA-binding capability.[8,9]

The mechanism of V(D)J recombination

Efficient cleavage of a DNA substrate requires only RAG1, RAG2, a divalent metal ion, and high-mobility group (HMGB1 or HMGB2) proteins. The first step in V(D)J recombination is binding of RAG, probably together with HMGB1 or HMGB2. RAG–RSS interactions have been studied. The RAG complex catalyzes two reactions, nicking and hairpin formation, without dissociation. First, RAG complex binds either a 12-RSS or a 23-RSS, resulting in a 12 or 23 signal complex. Then a nicking is introduced precisely at the junction between the coding segment and the RSS. Another type of RSS is captured to form a paired complex, and nicking is also follows.[10] *In vitro*, HMGB1 or HMGB2 have been shown to stimulate the activity of RAG in DNA binding, nicking and hairpin formation, presumably by inducing RSS bending.[9,11] Upon paired complex formation, the free 3′-hydroxyl released from the nicking step attacks the opposing strand

by transesterification in the presence of Mg^{2+} to create a hairpin coding segment and the cleaved RSS ends. Proteins in the non-homologous end joining (NHEJ) DNA repair pathway are recruited to the coding ends. The coding ends are opened by NHEJ DNA repair factors and then joined, forming imprecise coding joints that contain added nucleotides[9,12,13] (Fig. 1a). After V(D)J recombination of IgH and IgL chain genes, the resulting immature naive B cells transcribe the IgH and IgL genes and the transcripts undergo alternative splicing to produce IgD and IgM by fusing the μ and δ exons to the J exon.[1]

Non-homologous end joining is the major DSB rejoining process and occurs in all cell cycle stages, including two competing NHEJ pathways, canonic NHEJ (C-NHEJ) and alternative NHEJ (Alt-NHEJ; Fig. 2).

The mechanism of C-NHEJ has been well explained. The main proteins required for C-NHEJ involve the Ku70/Ku80 heterodimer, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), the endonuclease Artemis, the DNA ligase IV (Lig IV), the DNA polymerases μ (pol μ) and λ (pol λ), X-ray cross complementing Group 4 (XRCC4) and XRCC4-like factor (XLF).[14] Ku70/80 is thought to be the first protein to bind to the DSB, based on its abundance and tight affinity.[15] Ku70/80 protects DNA ends from digestion, and the Ku:DNA complex also serves as a node at which the nuclease, polymerases and ligase of C-NHEJ can attach. There is a Ku:DNA complex at each of the two DNA ends being joined, permitting each DNA end to be modified in preparation for joining. Ku:DNA complex recruits DNA-PKcs and Artemis, a DNA-PKcs-activated endonuclease, generating a DNA protein kinase (DNA-PK). DNA-PKcs undergoes autophosphorylation and activates Artemis, which then gains various nuclease activities

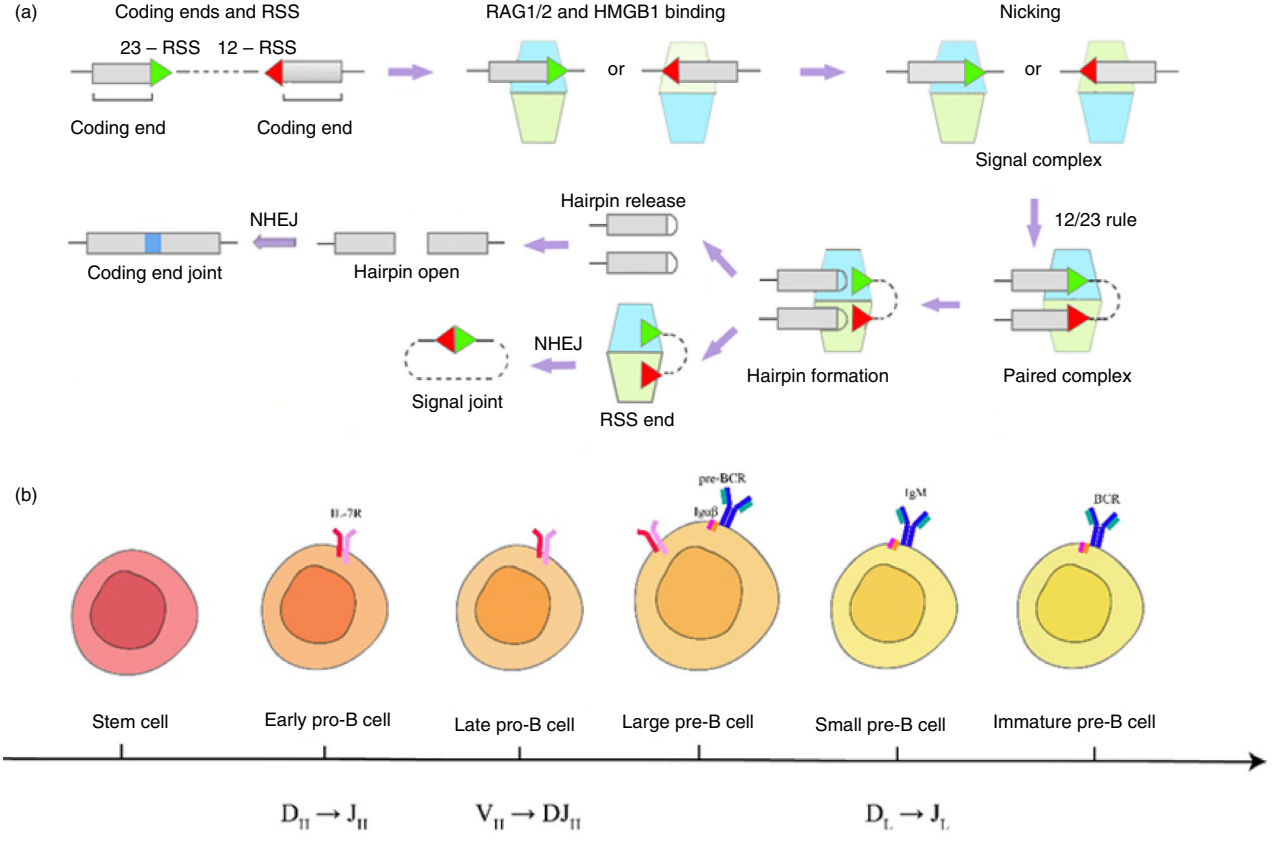

Figure 1. Overview of V(D)J recombination. (a) The RAG1–RAG2 complex is shown as light blue and light green trapezoids. Briefly, RAG binds a single recombination signal sequence (RSS) in the presence of HMGB1 to form a 12 or 23 signal complex. Another RSS is captured to form a paired complex. The DNA is cleaved, generating hairpin coding ends and RSS ends with a 3′ hydroxyl (OH) group in the presence of Mg^{2+}. The hairpin is released and opened and rejoined by non-homologous end joining (NHEJ), resulting in imprecise coding joints that contain added nucleotides (blue bars). RSS ends are processed through the NHEJ pathway as well, creating a signal joint. (b) B-cell ontogeny during V(D)J recombination. This figure shows the chronological order of B cells in different stages of development in the bone marrow. B cells progress from stem cells to pro-B cells, pre-B cells and immature pre-B-cell stages. During this differentiation, V(D)J rearrangement of heavy chains occurs in pro-B-cell periods, resulting in the expression of pre-B-cell receptors (pre-BCR), which are composed of Igμ and surrogate light chains (composed of VpreB and λ-5). After receiving the pre-BCR signaling, the light chains rearrange in small pre-B cells, resulting in the expression of a mature BCR (composed by rearranged heavy and light chains).

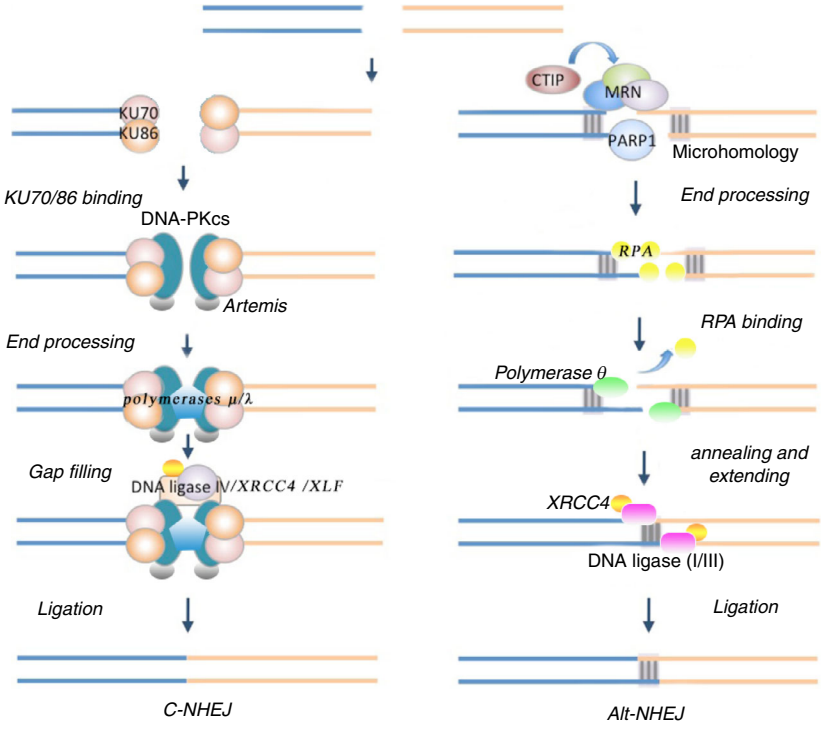

Figure 2. A model for canonical non-homologous end joining (C-NHEJ) and alternate NHEJ (Alt-NHEJ). C-NHEJ (left) and Alt-NHEJ (right) are shown. For C-NHEJ, Ku70/80 binds to the double strand breaks (DSB) first and protects DNA ends from digestion. The Ku:DNA complex recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in complex with Artemis, generating DNA-PK. DNA-PKcs undergoes autophosphorylation and activates Artemis, which then gains various nuclease activities, ensuring the DSB are compatible by resecting damaged DNA or non-ligatable end groups. The gap created is filled by Polμ/λ recreated by Ku. DNA-PK also facilitates recruitment of DNA ligase IV, XRCC4 and XLF to complete the ligation of DSB. For Alt-NHEJ, PARP1 recognizes the broken DNA ends and recruits Mre11-Rad50-Nbs1 (MRN) and CtIP complex to initiate 5′-3′ DNA resection, creating ssDNA overhangs. Resection exposes microhomology internal to break sites, facilitating spontaneous annealing of ssDNA. The binding of replication protein A (RPA) to the ssDNA overhangs removes secondary structure and prevents annealing of overhangs, which hinders Alt-NHEJ. Polθ-helicase acts as an ATP-dependent annealing helicase that dissociates RPA to promote DNA annealing and stimulate Alt-NHEJ. The paired ssDNA overhangs are subsequently extended by Po lθ-polymerase. Finally, end rejoining is carried out by the DNA ligase I or III (Lig I or Lig III)/XRCC1 complex.

including 5′-endonuclease activity, 3′-endonuclease activity, and hairpin opening activity.[15,16] The nuclease activities ensure that the two ends are compatible by resecting damaged or non-ligatable end groups at the end of the DSB. The gap created after processing is filled by Polμ/λ recruited by Ku.[17] DNA-PK also facilitates recruitment of a ligation complex, which encompasses LigIV, XRCC4 and XLF. PAXX (paralogue of XRCC4 and XLF) is a recently discovered protein with structural similarity to XRCC4 and XLF and promotes DSB repair by interacting with Ku.[18,19]

The role for Alt-NHEJ in V(D)J recombination was originally thought to be a backup pathway for DSB repair.[20] However, emerging evidence has demonstrated that Alt-NHEJ can also occur in C-NHEJ-intact cells.[20,21] The higher affinity of Ku for DSB compared with Poly-ADP ribose polymerase 1 (PARP1) could explain the predominance of C-NHEJ. The mechanism of Alt-NHEJ involves 5′–3′ resection of the DNA ends, annealing of microhomology, fill-in synthesis and ligation. PARP1 can recognize the broken DNA ends and serves as a scaffold for the recruitment of other DNA damage factors.[22] First, Mre11-Rad50-Nbs1 and CtIP complex initiates 5′–3′ DNA resection, creating short single-stranded DNA (ssDNA) overhangs.[20] Resection exposes microhomology internal to break sites, which could facilitate spontaneous annealing of ssDNA. The binding of Replication protein A (RPA) to the resulting short ssDNA overhangs removes secondary structure and prevents annealing of overhangs, which hinders Alt-NHEJ.[23,24] Polθ-helicase acts as an ATP-dependent annealing helicase that dissociates RPA to promote DNA annealing and stimulate Alt-NHEJ.[25] The paired ssDNA overhangs are subsequently extended by Polθ. Finally, end rejoining is carried out by the DNA ligase I or III (Lig I or Lig III)/XRCC1 complex in coordination.[22,26,27]

The recombination described above proceeds in such a way that intervening DNA is deleted. In fact, the

intervening DNA can also be inverted without deletion. Whether recombination proceeds in a deletional or inversional manner depends on the relative orientation of the two RSS. V and J segments located on the same strand are recombined by deletion, leaving the coding segment on the chromosome and the signal joint on an excised circle of DNA. If V and J segments are on opposite strands, they are joined by inversion between the RSS, generating a signal joint and a coding joint.

Notably, to generate an efficient immune repertoire, V(D)J recombination favors genetic diversity at two levels. The first level corresponds to a diverse rearrangement of V, D and J genes. The second level is characterized by a particular joining mechanism of coding segments characterized by the loss or addition of extra nucleotides. During V(D)J recombination, the terminal deoxynucleotidyl transferase adds random nucleotides (N-segments) at the V-D and D-J junctions in heavy chains of immunoglobulin, thereby significantly contributing to the diversity of the immune repertoire.[28]

Regulatory mechanism of V(D)J recombination

V(D)J recombination undergoes regulation at multiple levels, including the regulation of RAG1/2 activity, subnuclear reposition, spatial conformation of chromatin, and Vκ recombination, and has some specific regulation mechanisms.

The regulation of RAG1/RAG2 activity

The RAG are important enzymes in V(D)J recombination, so their activity and quantity have an important influence on this process. The RAG proteins can coordinate their activity through autoregulatory properties. The RAG1 N-terminus is also associated with ubiquitylation-dependent regulatory processes by acting as an E3 ligase.[29] RAG2 is a regulatory cofactor, which promotes RAG1 to bind and cleave DNA. However, with the RAG1 C-terminus (residues 1009–1040) collaboration, the C-terminus of RAG2 efficiently autoinhibits RAG activity.[30]

Phosphorylation events are related to the activity of RAG as well. RAG2 has a conserved serine-glutamine (SQ) phosphorylation site on 365 to 366. This ataxia telangiectasia mutated (ATM) -mediated phosphorylation site of RAG2 has the function of feedback control of cleavage and maintenance of genome stability.[31] Thr-490 is an essential Cyclin-dependent kinases phosphorylation site in a conserved degradation signal sequence, which links the amount of RAG2 to the cell cycle.[32] RAG activity can also be enhanced by the AMP-activated protein kinase, which directly phosphorylates RAG1 at serine-528. This phosphorylation event can increase the catalytic activity of the RAG, and so initiates V(D)J recombination.[33]

In addition, the sensitivity of the gene to RAG cleavage is related to the epigenetics of histone (see Supplementary material, Fig. S4).

The accessibility hypothesis postulates that the initial step of V(D)J recombination must be chromatin changes, which render the different genes of the immunoglobulin loci accessible to the V(D)J recombination machinery. The 3′ proximal region of immunoglobulin loci is activated by enhancer-mediated recruitment of histone-modifying enzymes. Consequently, J segments that are adjacent to the Eμ enhancer are characterized by an abundance of active histones like H3K4me2 (trimethylation of histone H3 at lysine 4), H3K4me3 and H3K9ac in pro-B cells. Through a plant homeodomain finger, the RAG2 proteins are able to specifically bind the active histone mark H3K4me3 in the RSS that are associated with J segments.[34] H3K4me3 subsequently changes the conformation of catalytic and substrate-binding regions of RAG1 and the autoinhibitory domain (residues 352 to 405) in RAG2, increasing substrate affinity and catalytic rate.[35,36] Additionally, SUV39H1, a histone lysine *N*-methyltransferase involved in the formation of heterochromatin, is involved in the establishment of H3K9me3 and H4K20me3, the hallmarks of heterochromatin. The formation of heterochromatin is known to influence RAG2, catalyzing the VDJ recombination. Moreover, SUV39H1 has a role in the methylation of RAG2, which changes RAG2 subnuclear localization, and might regulate the chromatin binding of RAG2.[37]

Apart from these ways to regulate RAG activity, there are also some mechanisms to regulate the amount of RAG. As RAG2 protein is only expressed in the G0-G1 phase, proteins that control the cell cycle can also influence the amount of RAG. The RNA-binding proteins ZFP36L1 and ZFP36L2 can suppress mRNAs, helping B-cell entry into and exit from the cell cycle. Cells that lack ZFP36L1 and ZFP36L2 show a delayed V(D)J recombination.[38]

Recent studies have shown that Forkhead box O (FOXO) protein has an important regulatory effect on the activity of RAG proteins (see Supplementary material, Fig. S5). Pre-B-cell receptors (pre-BCR) synergize with interleukin-7 receptor (IL-7R) signaling pathways, which is found to negatively regulate RAG mRNA and protein levels during the clonal expansion period of pre-BCR$^+$ cells, but positively regulates the expression of RAG in contrast to small pre-B-cell period.[39] FOXO protein is a major RAG transcription activator. It can activate the expression of RAG1 and RAG2, as well as up-regulate the amount of p27, a stabilizer for RAG2.[40] Phosphatidyl 3-kinase and Akt kinase, activated by tonic (foreign antigen-independent) receptor signaling, can phosphorylate and result in cytoplasmic separation of FOXO protein.[41] Moreover, early B-cell factor 1 (Ebf1), a transcription factor, can either directly regulate the accessibility of specific

genes at the loci of IgH and Igλ, or indirectly contributes to V(D)J recombination via the activation of Pax5.[42] The expression of Ebf1 is driven by the downstream effector Stat5 of IL-7R. During pre-B-cell proliferation, Ebf1 and its downstream target c-Myb have a negative influence on RAG transcription, through the negative regulation of FOXO1 binding to the RAG locus. Ebf1 can directly downgrade FOXO1 expression and upgrade zinc finger protein Gfi1b expression.[39,43] Similarly, activation of DNA damage is able to depress RAG expression by ATM-mediated canonical nuclear factor-κB transcription factors as well as the loss of ATM-dependent FOXO1 binding to the enhancer region of RAG1/2 locus (Erag).[43,44]

Subnuclear reposition

Evidence from several model systems has indicated that the location of genes, especially their proximity to heterochromatin, may be important for initiating or maintaining silence of a gene, and modulating locus accessibility. There are two repressive compartments, the nuclear periphery and pericentromeric heterochromatin, that are important for propagating the inactive state of genes. It has been revealed that subnuclear location will change during the development of a B cell.[45,46] In progenitors, the IgH locus is anchored at the nuclear periphery via the distal V_H genes that are in an accessibility repression stage;[47] meanwhile, the 3′ proximal D and J clusters are free, which facilitates D-J rearrangements in lymphoid progenitors. Both IgH alleles will undergo D-J recombination, which leads to a pro-B cell. IgH loci are subsequently relocated from the nuclear periphery to more central positions within the nucleus. This subnuclear repositioning reactivates V genes in preparation for V-DJ recombination. The two alleles behave differently at this stage of pre-B cells. Following successful V-DJ rearrangement of one IgH allele, which gives rise to pre-BCR, the signaling leads to repositioning of the second, incompletely rearranged IgH allele to repressive pericentromeric heterochromatin,[48] whereas the functionally rearranged IgH allele remains in a central position. This feedback inhibition of V-DJ recombination at the second allele ensures that each B lymphocyte expresses only one monospecific BCR, known as allelic exclusion (see Supplementary material, Fig. S6).[49] Mono-allelic expression seems to take place preferentially on the associated allele.[45] However, it has also been reported that activated B cells can transcribe both IgH alleles as well.[50] To what extent the two IgH alleles differ remains unclear.

Spatial conformation of chromatin

Chromatin interactions can control the gene assembly of IgH chains. In the pro-B cell, in which D-J recombination has finished but V-DJ recombination is going to take place, contraction will happen in the distal V cluster through looping.[48] The localized activity of the RAG1/2 at the proximal chromatin is strictly dependent on locus contraction for subsequent V(D)J recombination. The long-range contraction of the IgH locus also facilitates V-DJ rearrangements because of the proximity of both elements. In addition, different V genes can recombine with similar frequency, which is essential for the generation of a highly diverse immunoglobulin repertoire. Little is known about the molecular mechanisms controlling locus contraction and only a few *trans*-acting factors like Pax-5,[51] YY1[52] and CCCTC-binding factor (CTCF)[53,54] have been implicated in the regulation of contraction.[49]

Chromatin interactions control the gene assembly of IgH chains. In developing B lymphocytes, V(D)J recombination is also regulated by remote *cis*-acting elements. At distant sites of the IgH variable region, the 3′ regulatory region (3′RR) conveys a transcriptional silencing activity on transcription of the immunoglobulin gene, but the repressive activity was switched off after V-DJ recombination, promoting transcription of immunoglobulin in mature B cells.[55]

Besides, the highly conserved 11-zinc finger protein CTCF can mediate long-range chromatin interactions that influence the organization and function of the mammalian genome. CTCF also affects the complex configuration of chromatin loops that control the functional interactions between elements. CTCF binding site orientations can define the chromatin architecture that supports V(D)J recombination.[54]

In addition, histone deacetylase 3, a catalytic component of the NCoR/SMRT co-repressor complexes, is involved in global changes in chromatin structure, which likely have effects on distal V-DJ recombination.[56]

Vκ recombination regulation

The mechanisms of Vκ recombination regulation are different from V_H. First, the RSS for each V gene is different in quality, which can be quantified as RSS Information Content scores. Theoretically, a higher score indicates a tendency for recombination. In addition, Igκ recombination in pre-B cells is activated by the binding of some transcription factors like PU.1, E2A, IRF4 and IRF8 at the iEκ and 3′Eκ enhancers. PU.1 binding at the RSS is a key feature to determine whether the Vκ gene will actively recombine,[57] and the antisense lncRNA PU.1 is able to inhibit PU.1 mRNA translation by blocking tRNA recruitment.[58] In early B-cell progenitors, two DNA methyltransferases, Dnmt3a and Dnmt3b participate in the methylation of Tcfe2a gene, which encodes E2A. The lack of Dnmt3a and Dnmt3b elevates the level of E2A. Moreover, H3K4 methylation and IKAROS binding at the RSS are important for the promotion of higher recombination frequencies.[59,60]

Long-range genomic interactions, which distantly located genomic regions on the same chromosome undergo, are dynamically controlled at the Igκ light-chain locus. Yin Yang 1, a widely expressed transcription factor, contributes to long-range chromatin interactions in the IgH chain. Also, CTCF can regulate the expression of developmentally regulated genes. However, their roles in Igκ locus contraction have not been determined.[61,62] Recent research shows that a B-cell-specific enhancer, E88, is a major hub of long-range chromatin interactions, which connects subtopologically associated domains in the V gene region with a recombination center at the J genes in Igκ. This enhancer ensures a proper folding structure of Igκ locus for V(D)J recombination.[63]

The primary immunoglobulin repertoire is initially composed of IgM after the V(D)J recombination. The range of reactivity of the primary immunoglobulin repertoire can be further modified by somatic hypermutation (SHM) and class switch recombination (CSR) at the immunoglobulin loci. SHM results in mutations being introduced into the V region of the heavy chain and light chain, altering the affinity of the immunoglobulin for its antigen. Whereas in CSR, the initial heavy-chain C regions are replaced by another isotype, modifying the effector activity of the immunoglobulin but not its specificity. Both CSR and SHM are initiated by an enzyme called activation-induced cytidine deaminase (AID), which is expressed specifically in activated B cells. So these two processes do not occur in T-cell receptor genes.

Somatic hypermutation and class switch recombination

After going through V(D)J recombination, B cells subsequently undergo two genetic modifications, SHM and CSR. The purpose of these alterations, mostly in the germinal center, is to increase the affinity and alter the biological properties of immunoglobulin but with a specificity for the antigen. For some species, like chickens, immunoglobulin gene conversion is used in addition to or instead of SHM.[64] The molecular mechanism of SHM, CSR as well as gene conversion, shows many similarities and shares several enzymes. But AID is thought to be the only factor that is absolutely necessary for all these mechanisms. Below, the mechanism of SHM and CSR is described in detail.

AID is a 198-amino-acid protein. It is one of 12 members of the APOBEC family of DNA/RNA cytidine deaminases, and shares a conserved catalytic domain with other members. The catalytic domain contains His56, Cys87 and Cys90, which bind Zn^{2+} and are essential for catalytic activity, and Glu58, which serves as a general acid–base catalyst. The C-terminal domain is essential for AID to mediate CSR.[65] AID is a ssDNA-specific cytidine deaminase, which converts cytosine bases (C) to uracils (U) *in vitro* and *in vivo* but with no activity on dsDNA, RNA or RNA:DNA hybrids. The deamination reaction proceeds via a direct nucleophilic attack at position 4 of the pyrimidine ring of cytosine by Zn^{2+} coordinated to AID. However, AID is an inefficient enzyme, deaminating only about 3% of the cytidines even at preferred hotspot motifs. How this AID inefficiency effects CSR and SHM remains unclear at present.

Uracils caused by AID can trigger several types of DNA repair including the mismatch repair (MMR) and the base excision repair (BER) pathways. In the MMR, U mismatched with G is detected by the heterodimer MutSα, comprising MSH2 and MSH6.[66] MutSα recruits MutLα (MLH1-PMS2) and exonuclease 1 (Exo1). The endonuclease activity of PMS2 then cleaves the DNA 5′ of the mismatch, creating a DNA nick that serves as a point of entry for the Exo1, which initiates resection from the nick going past the mismatch site and creates an extended patch of ssDNA. Proliferating cell nuclear antigen (PCNA) subsequently recruits Polδ to replicate over the gap and Ligase 1 (Lig 1) finalizes repair. Whereas in BER, U is recognized by uracil-DNA glycosylase, which removes U from DNA[67], leaving an abasic site that is recognized by apurinic/apyrimidinic endonuclease 1/2 (APE1/2). APE1/2 nicks the DNA 5′ of the abasic site. PARP1 is activated and scaffold XRCC1, Polβ and a ligase are recruited. Polβ then removes the 5′ deoxyribose and inserts a single nucleotide, followed by a ligation and so completes the repair.[68] Interestingly, both uracil-DNA glycosylase and MutSα are conserved enzymes that initiate DNA repair with high fidelity through either MMR or BER. Hence, a non-canonical MMR or BER pathway is needed for transition or transversion mutations in SHM and DSB formation required for CSR (Fig. 3).

Mechanism of SHM

For error-prone MMR in SHM, APE2 rather than MutLα is recruited to nick the mismatch and provide an entry for the Exo1. Furthermore, low-fidelity polymerases like Polη instead of Polδ are recruited through PCNA ubiquitination[69] to resynthesize the DNA gap and introduce mutation, which is mainly at A:T residues. Whereas in non-canonical BER during SHM, some abasic sites serve as a non-informative template. REV, a Y-family DNA polymerase recruited by PCNA ubiquitination as well, inserts dCMP into the new DNA strand opposite the abasic site. After a further round of DNA replication this can result in a stable transversion mutation at the site of the original C:G base pair.

Mechanism of CSR

CSR is a DNA deletional-recombination reaction that proceeds through the generation of DSB in the switch (S) region preceding each C_H gene and is completed by

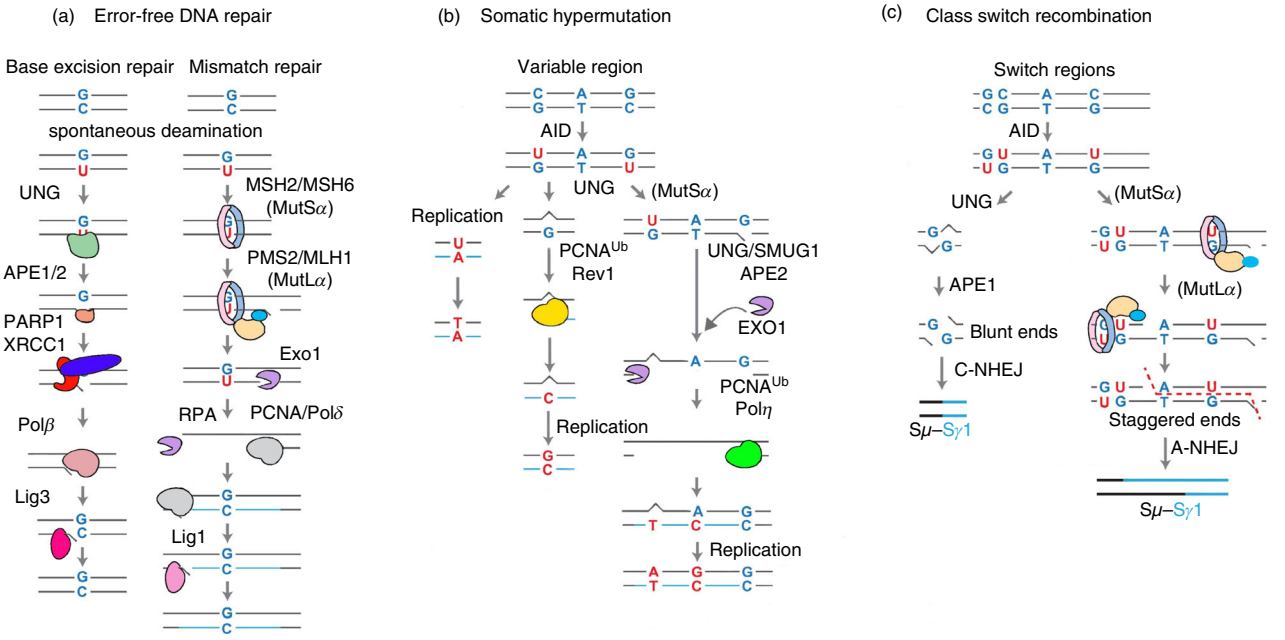

Figure 3. Overview of error-free basic excision repair (BER) and mismatch repair (MMR), somatic hypermutation (SHM) and class switching recombination (CSR). (a) Error-free repair pathways for BER (left) and MMR (right). Canonical BER is initiated by uracil-DNA glycosylase, creating an abasic site that is recognized by APE1/2. APE1/2 nick the DNA 5′ of the abasic site. PARP1 and XRCC1 are activated and recruit proliferating cell nuclear antigen (PCNA) and polymerase *β* (Pol*β*). Pol*β* removes the remaining 5′ deoxyribose and insert a single nucleotide, followed by ligation with Lig3. In MMR, MutSα recognizes the U:G mispair and recruits MutLα. MutLα nicks the DNA 5′ of the mismatch via PMS2. Exo1 creates an ssDNA from the nick going past the mismatch site. PCNA subsequently recruits Pol*δ* to fill over the gap, following ligation by Lig1. (b) During SHM, uracil can act as a template for replication leading to a C–T transition mutation. Alternatively, non-canonical BER or MMR recruit low-fidelity polymerases Pol*η* through PCNA ubiquitination (PCNA-Ub) leads to transition or transversion mutations. (c) For CSR, non-canonical BER can lead to double strand breaks (DSB) when two uracils in opposite strands are closely spaced. MMR can process distantly spaced uracils, leading to staggered DSB. Blunt DSB are joined by canonical non-homologous end joining, whereas staggered breaks are repaired by alternative non-homologous end joining.

NHEJ between donor and acceptor S regions, resulting in the replacement of the expressed C_H exon cluster.

The S regions are repetitive DNA elements that locate upstream of all the C_H genes except C*δ*. Mouse S regions are composed of 1–10 kb of tandem repeats that are G-rich on the non-template strand. Both the sequence and number of tandem repeats vary among mouse S regions. S*μ*, for example, is approximately 3·2 kb long and is comprised of GAGCT pentameric motifs, with the AGCT palindromic sequence representing a canonical RGYW/WRCY sequence. S*γ*1 is approximately 10 kb long and has multiple RGYW/WRCY sequences embedded within 49-bp repeat units. Other repeat motifs found in S regions include GGGGA/T in S*μ*, S*γ*1, S*γ*2b and S*γ*3 and GGGCT in S*ε* and S*α*. S regions are the targets for AID that initiates CSR.[65,70]

There are a transcriptional intervening (I) promoter and an I exon upstream of all C genes except C*δ*. At the onset of CSR, I promoters are selectively activated and produce non-coding germline transcripts, which initiate at the I exon, proceed through the S region and terminate downstream of the corresponding C_H gene.[71] AID is a ssDNA-specific cytidine deaminase, so germline transcripts are thought to separate the two dsDNA and provide access to AID.[72] AID binds to the S region by 14-3-3 adaptors[73] and recruits protein kinase A as well. AID is phosphorylated at Ser38 of the N-terminal region by protein kinase A, generating a binding site for RPA.[74] RPA enhances the deamination activity of AID and transfers C into U. If two U are sufficiently near in opposite strands, a DSB is resulted through the BER pathway. Conversion of two more distal U into a blunt DSB, which is necessary for CSR, requires MMR.[75]

AID-initiated DSB in two S regions activate the ATM-dependent DNA damage response. The Mre11-Rad50-Nbs1 complex is first recruited to a DSB, where it recruits the protein kinase ATM. ATM phosphorylates and recruits numerous DNA damage response proteins including H2AX, Mediator of Damage Checkpoint protein (MDC1),[76] ubiquitin ligases RNF8 and RNF168, p53-binding protein 1 (53BP1), breast cancer 1 (BRCA1) and receptor-associated protein 80 (RAP80).[77] H2AX and 53BP1 mediate synapsis of upstream and downstream DSB,[78] and 53BP1 can also prevent the rejoining of intra

S-region DSB.[79] DSB in different S regions are eventually rejoined by C-NHEJ or Alt-NHEJ. Both C-NHEJ and Alt-NHEJ pathways are used in CSR.[80] DSB generated by the MMR pathway seem to favor Alt-NHEJ, possibly by exposing microhomologies after Exo processing.[75]

IgD CSR is restricted to a few B-cell subsets in specific lymphoid tissues such as mesenteric lymph nodes, peritoneal cavity and mucosa-associated tissue in both mice and humans.[81–83] A recent study suggested that IgD CSR is initiated by microbiota,[83] implying a role of IgD in the regulation of microbial homeostasis.

Locus suicide recombination

Immunoglobulin production relies on the selection of antigen-specific B cells. This implies not only proliferation and differentiation of those B cells that specifically bind antigen but also elimination of the less efficient or inappropriately activated cells.[84] It is important for B cells to undergo such a choice as inappropriate production of useless cells might trigger autoimmunity and inflammation. Locus suicide recombination (LSR) is reported as a variant form of the recombination mechanism to eliminate inappropriate B cells. LSR is characterized by recombination between Sμ and the 3′RR downstream of the C region, deleting the whole IgH constant gene cluster and thereby terminating B-cell function. Apart from direct junctions from Sμ to 3′RR, sequence analysis of LSR both *in vivo* and *in vitro* reveals the presence of junctions of Sγ or Sα regions rather than Sμ with 3′RR, indicating that LSR has taken place in previous CSR in at least 14% of cases.[85] Murine 3′RR includes repetitive DNA sequences resembling S regions. In humans, there are two 3′RRs due to the interval duplication of Cα gene on the IgH locus (3′RR1 and 3′RR2, respectively, downstream of Cα1 and Cα2).[86] Analyzing the structure of human 3′RR shows their high similarity with the mouse 3′RR and both of them share multiple features with S regions. These 'Like switch' regions promote LSR through a process that is similar to CSR: non-coding germline transcription of Sμ and 'Like switch' within the 3′RR, DSB caused by AID, and finally DNA repair, mainly by the NHEJ pathway as in CSR.[87] However, major involvement of the A-NHEJ rather than C-NHEJ during DSB repair in LSR compared with CSR was recently reported,[88] suggesting a mechanism to control the balance between CSR and LSR. LSR is mostly detected during B-cell activation but remains undetectable in long-lived memory cells or plasma cells.[85] Interestingly, as LSR is a process towards cell death, it remains difficult to evaluate its regulation.[85]

Regulation of AID

Given that AID plays a significant role in both CSR and SHM, it is a crucial mechanism to regulate CSR and SHM by modulating AID expression, stability and localization.

Epigenetic regulation of AID

Histone modification and miRNA are two significant epigenetic mechanisms that regulate gene expression. In B-cell activation, stimulatory signals induce several histone-modifying enzymes to active H3K4me3, H3K9ac and H3K14ac in the promoter regions of AID and remove the repressive H3K27me3 and H3K9me3, changing the chromatin structure or recruiting other modifying factors and so up-regulating AID. These signals also activate miRNA like mir-16, mir-155 and mir-181b that decrease the expression of AID by binding to and degrading complementary sequences of the mRNA.[89]

Phosphorylation also modulates AID activity at a post-translation level. Between 5 and 15% of the AIDs in activated B cells are phosphorylated at serine 38 by protein kinase A, and it has a role in targeting AID to DNA and increasing its activity.[90] Moreover, threonine 140 can also be phosphorylated by PKC family members, enhancing AID relatively. Threonine 140 phosphorylation preferentially affects SHM, suggesting that post-translational modifications may contribute to the choice between CSR and SHM.[91] Serine 3 is another phosphorylation point controlled by protein phosphatase 2. Increasing S3 phosphorylation leads to a reduced activity of AID.[92]

The localization and stability of AID

It is contradictory that AID is predominantly located in the cytoplasm whereas both CSR and SHM occur in nucleus. Further studies have revealed that AID is transported between nucleus and cytoplasm continuously, although the mechanism for this is not clear. Germinal center-associated nuclear protein, an RNA-binding protein, is assumed to transport AID into the nucleus in mice.[93] Cytoplasmic AID interacts with eukaryotic elongation factor 1α (eEF1A), a translation elongation factor involved in AID cytoplasmic retention.[94] Export of AID is mediated by the Ran-dependent nuclear exportin CRM1 because AID has a powerful nuclear export signal at the C terminus.[95]

Localization of AID is also associated with its stability. In the nucleus, AID undergoes a ubiquitin-dependent protein degradation as well as a Reg-γ-mediated ubiquitin-independent degradation.[96] Whereas in the cytoplasm, molecular chaperones like Hsp90 and DnaJa1 can inhibit the polyubiquitination and proteasomal degradation of AID, and so increase the stability of AID.[97] The complex regulatory mechanism of AID subcellular localization and stability allows proper amounts of AID to access into the genome, protects the genome from continuous mutations, and minimizes the detrimental off-target effects.

Bach2

BTB and CNC homology 2 (Bach2) is a B-cell-specific transcription repressor and is thought to be the common pathway in controlling the expression, protein stability and subcellular location of AID.[98] Bach2 up-regulates AID mainly by suppressing B lymphocyte-induced maturation protein-1 (Blimp-1), a factor that drives plasma differentiation by suppressing mature B-cell-associated genes including AID.[99] Bcl6 can co-bind to the *cis*-regulatory sequence of the Blimp-1 gene with Bach2, up-regulating AID and maintaining the stability of the Bach2.[100] However, Bcl6 as well as other suppressors of Blimp-1 like Pax5 and Spib can be in turn blocked by Blimp-1 at a transcriptional level, forming a negative feedback regulatory pathway.[101] Moreover, Bach2 can also upgrade the expression of AID through a Blimp-1-independent pathway.[98] Apart from a decreased amount of AID in B cells, Bach2 deficiency also profoundly decreased AID level in the nucleus.[98] Bach2-deficient cells also had reduced expression of the molecular chaperones known to stabilize cytoplasmic AID.[102]

Other regulatory mechanism of CSR

3′ regulatory region

The 3′RR is located at the most distal 3′ region of the immunoglobulin H chain locus and comprises four hypersensitive sites: hs3a, hs1,2, hs3b and hs4. Each hs is a weak enhancer but they form a strong enhancer synergistically.[72,103] The 3′RR is crucial for conventional CSR,[82,104] but its mechanism remains unclear. Conclusions drawn from knock-out mice are that the 3′RR plays a role in promoting germline transcription[105,106] and synapsis between S regions in CSR.[107] In addition, it has also been reported that 3′RR mainly controls the S acceptor region rather than the Sμ donor region in multiple aspects including alterations of epigenetic marks, germline transcription, R-loop formation, paused RNA PolII, AID targeting and generation of DSB. Once DSB are generated, the Sμ–Sγ1 junctions are not affected by the 3′RR.[108] The complete 3′RR deletion dramatically affects CSR and immunoglobulin secretion for all isotypes.[104] But the IgG1 class displays a special status as only Cγ1 transcription and CSR are partly preserved after alteration or even complete deletion of the 3′RR, whereas all other class-switched immunoglobulin was nearly abolished, suggesting that other elements may support the γ1 transcription.[104–106] Furthermore, it seems that IgD CSR[109] and IgA CSR in B1 cells[110] are totally independent on the 3′RR. The Sμ–$\sigma\delta$ junction is mainly produced by A-NHEJ rather than C-NHEJ,[109] which markedly differs from IgG, IgA and IgE CSR.

Chromosome looping

The activation of CSR likely needs the signal from BCR, and the secondary signals from CD40 and Toll-like receptors. Next, the transcription of unrearranged C_H genes regulates naive B cells to a specific isotype. The type of cytokines can regulate the direction of CSR by chromosome looping, as different transcription promoters, located upstream of each acceptor S region, are activated by different cytokines. Then specific S-C loci (like Sε-Cε loci) are closed to the Eμ-Sμ-Cμ and 3′ Cα enhancer (3′Eα) region. Finally, the formation of chromosome looping can induce cell switching to that specific isotype (Fig. 4).[111]

G-quadruplex

G-quadruplexes (G4) are secondary structures that are formed in nucleic acids by guanine-rich sequences. They are constructed around continuous G-tetrads of Hoogsteen hydrogen-bond guanine bases.[112] G4 DNA structures have been previously reported to be present on the non-template DNA strand of Sμ and Sγ regions *in vitro*. Recent studies show that AID deaminates ssDNA more robustly in the context of structured substrates, such as G4. Some studies have shown that G4 formation plays a role in CSR. G4 mediates recruitment and oligomerization of AID, which helps to create mutation clusters and DSB on IgH chain.[73] Conversely, G4-stabilizing agents, like (3,11-difluoro-6,8,13-trimethyl-8H-quino(4,3,2-kl)acridinium), can inhibit CSR and decrease immunoglobulin secretion.[113] Moreover, two nucleoside diphosphate kinase (NME) isoforms are novel players in the CSR process. Before CSR activation, NME1 binds to the S region and suppresses CSR. Upon stimulation, NME1 dissociates from activated S region, whereas NME2 binds to G4 in the S region to promote CSR. NME1 and NME2 act as coordinated inhibitory stimulus pairs to modulate CSR.[114]

R-loop

R-loop is a stable intermediate of CSR, which is composed of a DNA:RNA hybrid and the associated non-template ssDNA. R-loop forms behind the extended RNA Pol II in the S region and results in ssDNA that may act as a substrate for AID, the ssDNA-specific cytidine deaminase. In recent years, many studies have found that, compared with the original model, R-loop is unlikely to play a role in the initial AID-dependent mutagenesis phase, and it must have other AID-independent roles in CSR. For example, R-loop can regulate the DNA replication environment at the IgH locus.[115,116]

Many other factors participate in the regulation of CSR through the R-loop pathway. DEAD-box RNA helicase 1 (DDX1) is a DEAD-box RNA helicase required at the IgH locus for CSR *in vivo*. DDX1 binds to G4 structures,

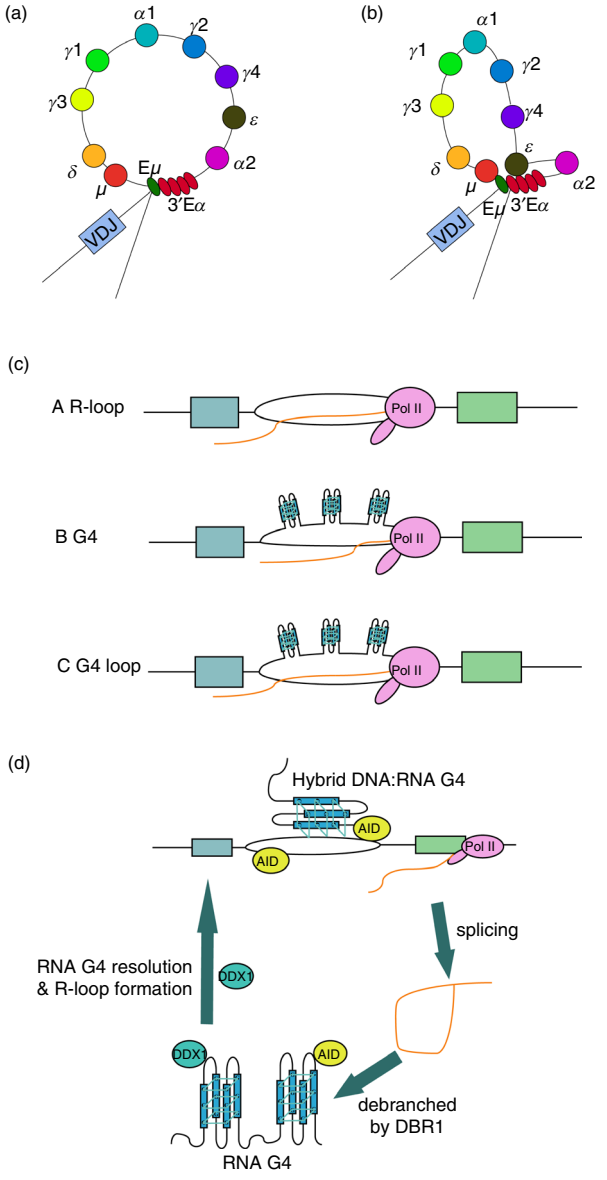

Figure 4. Chromosome looping and R-loop. (a) This picture shows the structure of the chromosome looping, and each colorful circle representing the specific S-C loci. Eμ and 3′Eα interact, causing Sμ and the downstream S regions located in a chromosomal loop. (b) After the Igε transcription promoter (located at the upstream of Igε acceptor S region) activated, Sε-Cε loci are close to the 3′Eα segment and Eμ-Sμ-Cμ, inducing the B cell to switch to IgE. (c) Structures that recruit activation-induced cytidine deaminase (AID): the switch sequence has a high G-richness on the non-template chain. During transcription, these G-rich regions form secondary structures such as the R-loop, G-quadruplexes (G4) and G-loop, which help recruit AIDs in the S region. (d) DDX1 promotes R-loop formation: RNA polymerase II (Pol II) produces switch transcripts, whose intron contains the G4. After a splicing step, the intron lariat intermediate is debranched by RNA lariat (intron) debranching (DBR1) enzyme. Bound G4–AID complex, DDX1 targets to the S-region DNA. At last G4 is resolved, and an R-loop is formed in the S region.

converting them into an S-region R-loop. This process targets AID to the S regions, resulting in the promotion of CSR (Fig. 4d),[116] which is how DDX1 promotes R-loop formation.

Other regulatory pathways

Many other molecules have been discovered that regulate CSR. For example, histone methyltransferase and multiple myeloma SET domain can promote AID-mediated DNA breaks during CSR.[117] CD11b, an integrin expressed on the surface of activated B2 cells induces AID expression by the nuclear factor-κB signaling pathway.[118] Estrogen receptors (ER) in activated B cells may control CSR by the direct binding of ER to key regulatory elements in the IgH chain locus, such as Sμ, Eμ and the 3′RR.[119] basic leucine zipper transcription factor, ATF-like promotes the production of class-switched immunoglobulin by positively regulating the expression of Nfil3 and miR155hg, but negatively regulating Wnt10a.[120] The BER enzyme methyl-CpG binding domain protein 4 (Mbd4) participates in MMR-directed DNA end processing by interacting with MutL homologue 1 (MLH1).[121] The nuclear structural protein NuMA interacts with 53BP1, controlling 53BP1 diffusion and negatively regulating 53BP1 in DSB repair.[122] The serine/threonine phosphatase PP4 shows a promoting component of CSR by involvement in sequential recruitment of RPA and NBS1.[123]

Immunoglobulin-specific isoform induction

In addition to the mechanisms above, certain conditions can also induce immunoglobulin-specific isoform production. Isotype switch recombination is controlled by cytokine receptors and their downstream signals. It is widely believed that IgG1 and IgG3 are mainly produced in response to protein antigens, but chronic stimulation with these antigens leads to an increase in the proportion of IgG4. Interleukin-4 can induce IgE and IgG secretion whereas IL-10 can induce the production of IgG1 or IgG3. Transforming growth factor-β is involved in the conversion to IgA, and interferon-γ can induce the production of IgG2a or IgG3.[124,125]

There are many newly discovered inducing factors for IgE with a lower activation threshold, so compared with IgG1, IgE has a higher transcription probability in the early choice.[126] Thymic stromal lymphopoietin from damaged epithelial cells following allergen stimulation can activate dendritic cells, triggering naive $CD4^+$ T cells to differentiate into inflammatory T helper type 2 effector cells and secrete the cytokines of IL-4, IL-9 and IL-13, which enhance IgE production. Dendritic cells also activate allergen-specific T helper type 2 memory

cells, helping immunoglobulin switches from IgM to IgE.[127] In addition, microRNA-146a up-regulates the expression of 14-3-3σ , which is a key protein of CSR. This process enhances class switch and secretion of IgE in B cells.[128]

There have also been some recent discoveries about IgG and IgA CSR. Specific inhibitors of elastase cause cells to secrete higher levels of IgG and IgA in mice, and increase transcription of factors involved in murine B-cell differentiation, like mRNA for AID, IL-10, B-cell-activating factor of the tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL).[129] Dectin-1 agonists, like heat-killed *Saccharomyces cerevisiae*, can selectively induce IgG1 class switching in lipopolysaccharide-activated B cells through reinforcing surface expression and direct stimulation of Dectin-1.[130]

Conclusion

Given the importance of the diverse repertoire of immunoglobulin in immune responses, it is reasonable to suppose that sophisticated genetic mechanisms have evolved to generate and regulate these proteins. However, these mechanisms are still not fully elucidated. After more than one century of research, it has been discovered that the generation of immunoglobulin undergoes a series of specific genetic events including V(D)J recombination, CSR and SHM. V(D)J recombination contributes to the generation of surprising immunoglobulin diversity. CSR and SHM can increase the affinity of immunoglobulin to recognize and bind with various antigens. Even though the molecular mechanisms of V(D)J recombination, CSR and SHM have been studied in detail, much remains unclear. For example, how inefficient AID initiates CSR and SHM, how CSR and SHM are well regulated and compete with error-free DNA repair pathways, and how B cells regulate LSR are still not well understood. Identification of regulatory factors and their function in V(D)J recombination, SHM and CSR, especially those associated with RAG and AID, do require further elucidation.

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Disclosures

The authors declare no financial or commercial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunoglobulin gene locus and IgG structure.

Figure S2. RSS sequence and location.

Figure S3. RAG1/2 structure and function.

Figure S4. Histone modification and contraction of V cluster.

Figure S5. Molecules that regulate the amount of RAG by FOXO.

Figure S6. Subnuclear location of the IgH locus.