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The B cell dilemma: Diversity or fidelity?

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ABSTRACT

The ability of B lymphocytes to diversify immunoglobulin (*Ig*) genes is central to the generation of high-affinity, class-switched antibodies and the establishment of effective humoral immunity. This diversification is achieved through three DNA remodeling processes that occur at defined stages of B cell development and maturation: V(D)J recombination, somatic hypermutation (SHM), and class switch recombination (CSR). These reactions all rely on the induction of programmed DNA lesions at *Ig* genes and their productive resolution by ubiquitous DNA repair pathways. However, such physiological sources of genotoxic stress render B cells vulnerable to genome instability, including mutations and chromosomal translocations that drive malignancies. Therefore, B cells have evolved complex regulatory networks that ensure efficient *Ig* gene diversification while minimizing the risk of unproductive or deleterious repair outcomes. In this review, we integrate foundational studies with recent mechanistic advances to outline how B cells exploit, coordinate, and constrain DNA repair to balance immune receptor diversification with the preservation of genome integrity.

1. Antibody gene diversification during B cell development and maturation

The establishment of effective immune responses against foreign antigens relies on the generation of highly specific antibodies that neutralize, opsonize, and/or facilitate the clearance of these threats [1,2]. This branch of the adaptive immune system is referred to as humoral immunity. Defects in the molecular and cellular processes underlying humoral responses lead to increased susceptibility to infections, immunodeficiencies, and autoimmune conditions, as well as lymphoproliferative disorders and poor vaccine responses [3,4].

Because of their ability to produce and diversify the antibody repertoire, B lymphocytes are the primary mediators of humoral immunity [1,2]. Antibodies represent the secreted forms of immunoglobulins (Ig) and are composed of two identical Ig heavy and light chains, which together form a variable domain, responsible for the antigen specificity, and a constant domain, which defines the antibody's effector function (Fig. 1A). The Ig chains are expressed from distinct loci in the B cell genome (Ig heavy, *IgH/h*, and light, *IgL/l*) (Fig. 1B), and both the generation and refinement of the antibody repertoire occur *via Ig* loci diversification reactions that are tightly linked to key developmental and maturation stages of the B cell lineage [5] (Figs. 1B and 2).

B lymphocytes originate in the bone marrow *via* a sequence of developmental steps, the progression of which relies on the proper assembly of the membrane-bound form of the Igs, the B cell receptor (BCR) [6] (Figs. 1A and 2). For successful development, the BCR must not only be correctly expressed on the cell surface but also capable of transmitting signals effectively. Developing B lymphocytes whose receptors fail to generate adequate activation signals are unable to progress further along the developmental trajectory [7,8]. During the initial pro-B cell stage, the gene encoding for the variable region of the heavy chain locus is assembled through stepwise rearrangements of first, a diversity (*D*) with a joining (*J*) gene segment, followed by a variable (*V*) element, from the pools of available segments in the germ-line configuration [7,8] (Figs. 1B and 2). This primary Ig diversification reaction, known as V(D)J recombination, occurs *via* the developmentally programmed formation and repair of DNA double-strand breaks (DSBs) between the recombining gene elements [9] (Fig. 1B). Association of the newly rearranged heavy chain with the surrogate light chain (SL), composed of λ5 (lambda-5) and VpreB proteins, enables the surface expression of the pre-B cell receptor (pre-BCR) on pro-B cells [10] (Fig. 2). As a consequence, pre-BCR-mediated survival and proliferation

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signals promote further B cell development to the subsequent pre-B cell stage, where the light chain locus undergoes VJ recombination [10] (Fig. 2). Association of the heavy chain and the recombined light chain leads to the expression of a mature BCR, which marks the completion of antigen receptor assembly and enables the cell to exit the bone marrow as an immature B cell (Fig. 2). These cells pass through transitional stages in peripheral lymphoid organs, where they complete their development into naïve B cells and remain in a resting state [11] (Fig. 2).

Activation of mature naïve B cells typically occurs within secondary lymphoid tissues, such as lymph nodes or the spleen, in response to antigen engagement and cognate help from follicular helper T cells [1]. This activation leads to the formation of germinal centers (GCs), which are specialized microenvironments within B cell follicles where affinity maturation, isotype switching, and differentiation into effector B cell subsets occur [1] (Fig. 2). Within the dark zone of the GCs, B cells proliferate extensively and undergo somatic hypermutation (SHM) of their *Ig* variable region genes at both *Igh* and *Igl* loci [12] (Figs. 1B and 2), a process that introduces point mutations at a high frequency and is essential for generating antibodies with increased affinity for antigens. Class switch recombination (CSR) occurs mainly at the pre- or early GC stage and enables B cells to replace the constant domain of the initially expressed Igh chain isotypes IgM and IgD with those corresponding to the other classes, IgG, IgE, or IgA, thereby altering the antibody's effector function while preserving antigen specificity [1,13] (Figs. 1B and 2). Analogously to V(D)J Recombination, CSR occurs *via* the tightly-regulated formation and repair of DSBs at the *Igh* locus [14] (Fig. 1B).

B cells that successfully acquire high-affinity BCRs through SHM are

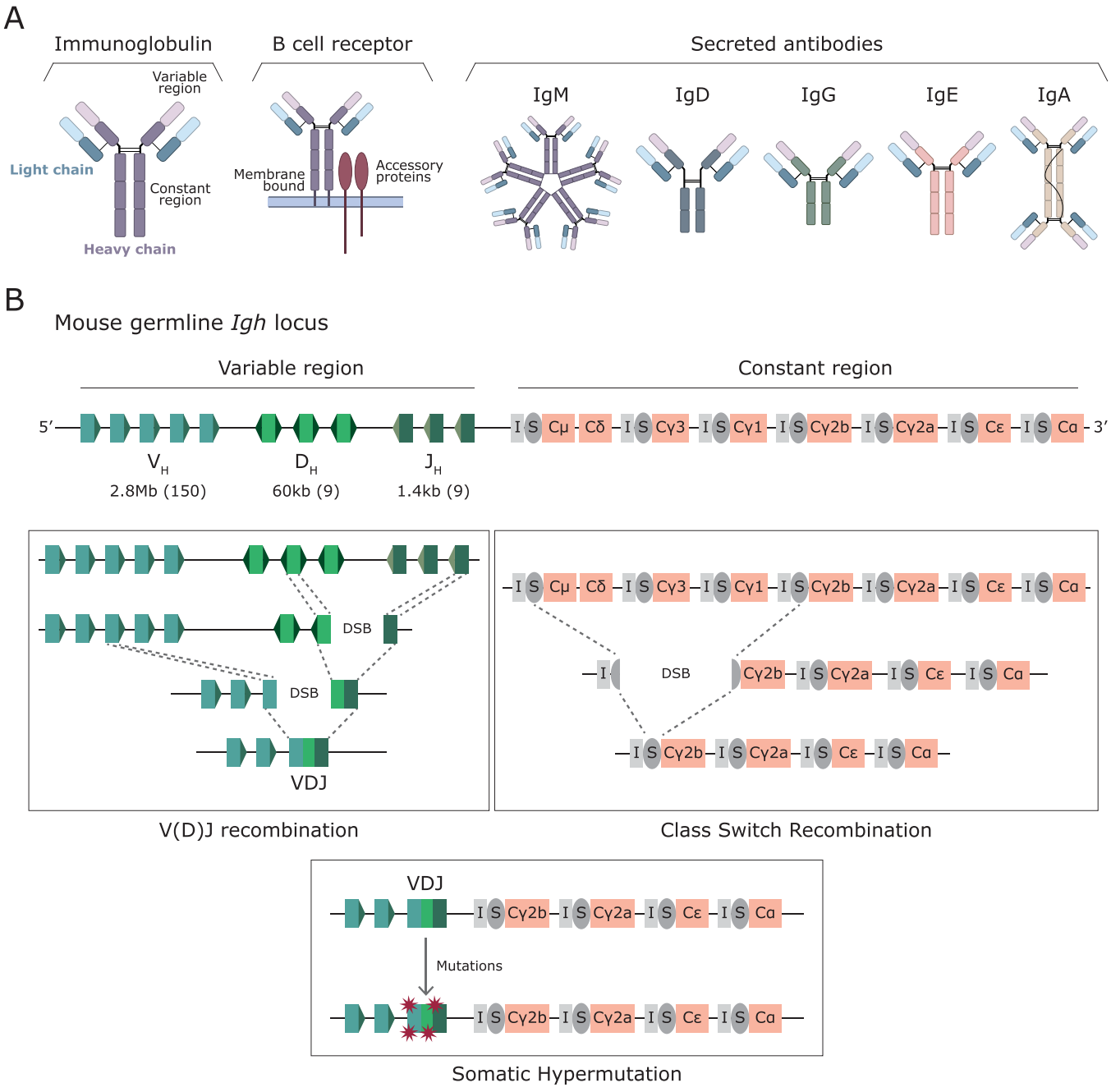

Fig. 1. Immunoglobulin gene diversification reactions. A) Schematic representation of an immunoglobulin molecule (left), its membrane-bound form (B cell receptor, BCR) (middle), and the different secreted isotypes (right). B) The mouse *Igh* locus shown in its germline configuration (top) and its rearranged states following V(D)J recombination, CSR, and SHM (bottom). The approximate number of gene segments in V_H, D_H and J_H regions is shown in parentheses.

positively selected in the light zone of the GCs based on their ability to bind antigen and receive survival signals from follicular T helper cells [1]. These positively selected B cells then differentiate into either antibody-secreting plasma cells or long-lived memory B cells [1] (Fig. 2). Plasma cells, particularly those that home to the bone marrow, are responsible for sustained antibody production and long-term systemic protection. Memory B cells, on the other hand, provide rapid and robust responses upon re-exposure to antigen, often with enhanced affinity and isotype diversity due to prior GC experience [1]. Together, these tightly regulated maturation processes following bone marrow egress ensure the generation of a highly specific, adaptable, and durable humoral immune response.

While V(D)J recombination occurs in both B and T lymphocytes, CSR and SHM are exclusive to B cells and play distinct, yet complementary, roles in shaping the antibody repertoire. However, the programmed DNA alterations that these reactions rely upon inherently threaten genomic stability. Failure to accurately resolve these DNA lesions not only impairs genome diversification and protective immunity, but can also result in chromosomal translocations and mutations, which are all hallmarks of lymphoid malignancies [4]. Therefore, throughout their development and maturation, B cells endure a uniquely high level of programmed genotoxic stress, far exceeding that of other somatic cell types, and must continuously maintain a delicate equilibrium between enabling antigen receptor diversification while rigorously safeguarding the genome against oncogenic events. Although the fundamental mechanisms of receptor gene diversification and humoral immunity are conserved across mammalian species, we primarily refer to the mouse model for gene nomenclature and mechanistic details, given that most recent insights stem from studies conducted in mice.

2. Keeping V(D)J recombination in check

V(D)J recombination is initiated by the lymphoid-specific recombination-activating genes RAG1 and RAG2, collectively referred to as RAG, which introduce site-specific DSBs at recombination signal sequences (RSS) flanking the *V*, (*D*) and *J* segments of both the *Igl* and *Igh* loci [15–17] (Fig. 3). RSSs are non-coding DNA elements characterized by conserved heptamer (7 bp) and nonamer (9 bp) motifs, separated by a less conserved spacer of either 12 or 23 base pairs, referred to as 12-RSS or 23-RSS, respectively [15–17] (Fig. 3). RAG binding of a 12-RSS and a 23-RSS, a pairing requirement known as the 12/23 rule, and subsequent cleavage result in two distinct types of DNA ends: hairpin-sealed coding ends and blunt signal ends. While coding ends are processed to eventually form coding joints, signal ends are joined together to produce signal joints [15–17] (Fig. 3). The resolution of both of these DNA intermediates during V(D)J recombination is primarily mediated by the DSB repair pathway non-homologous end joining (NHEJ) [15–17]. The high mobility group box proteins HMGB1 and HMGB2 are also recruited to facilitate the formation of the paired complex, thus permitting the subsequent DNA cleavage by the RAG [18–20] (Fig. 3). Genetic mutations affecting the cleavage, processing, or joining of coding ends give rise to a range of immunodeficiency

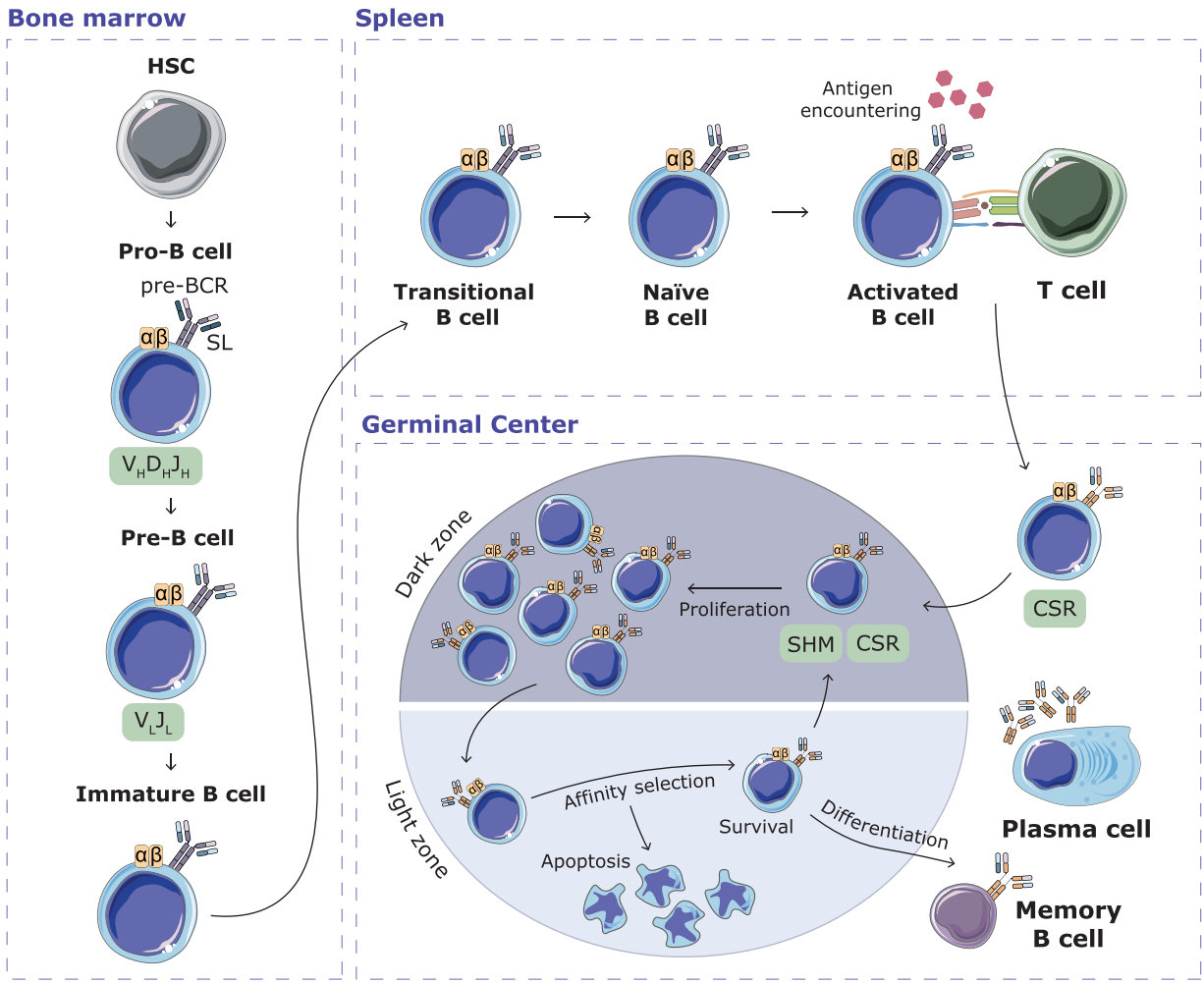

Fig. 2. B lymphocyte development is a multistep process tightly linked to antibody gene diversification. Schematic representation of B cell development from hematopoietic stem cells (HSCs) in the bone marrow to antigen-dependent maturation in secondary lymphoid organs.

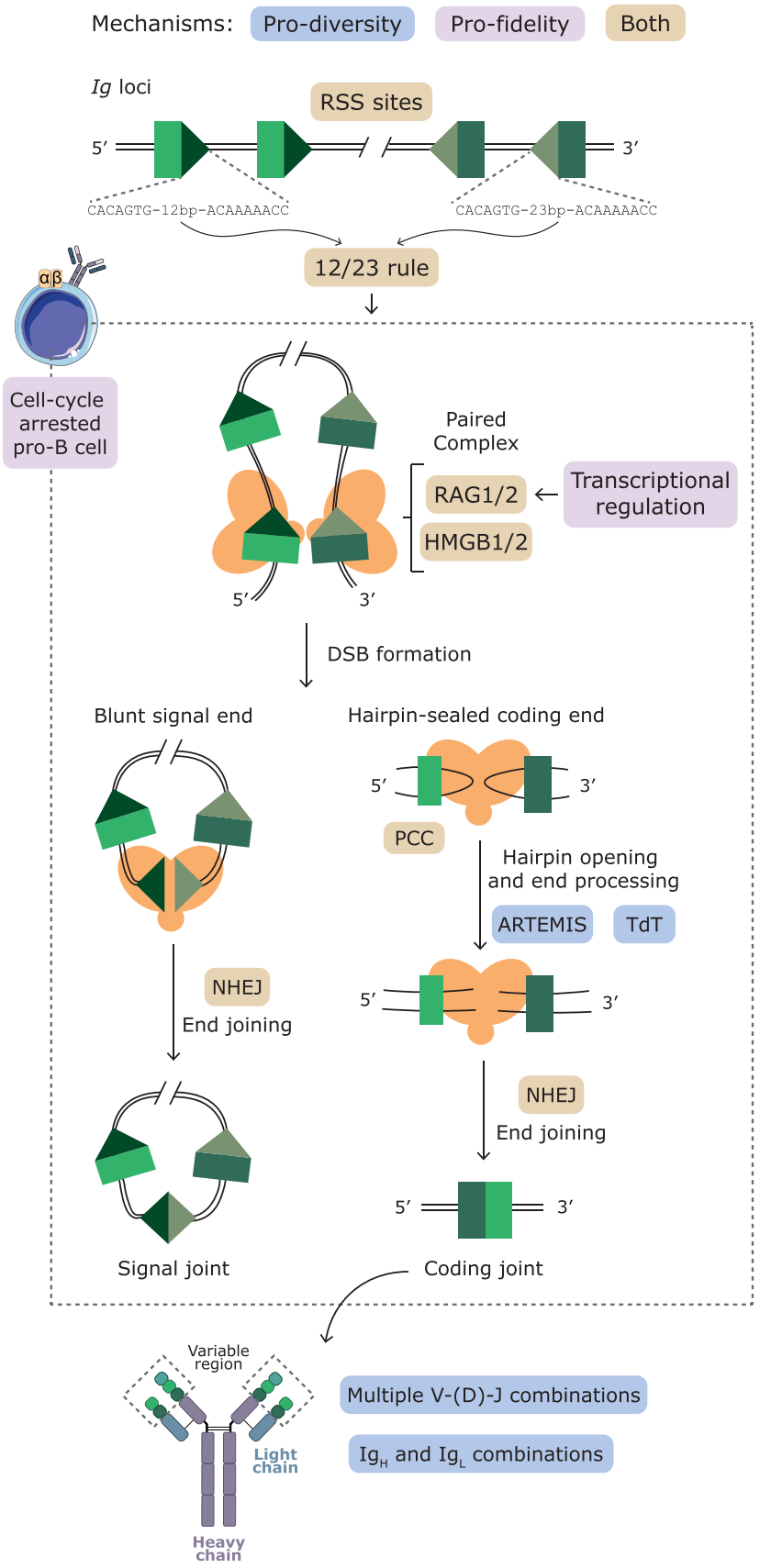

Fig. 3. Mechanisms balancing diversification and genome integrity during V(D)J recombination. Schematic overview of V(D)J recombination highlighting the mechanisms that promote antibody diversity (pro-diversity), preserve genome integrity (pro-fidelity), or contribute to both.

syndromes collectively classified as severe combined immunodeficiency (SCID) [21–23].

Although the initiation of DSB formation during V(D)J recombination requires stringent conditions for site-specific cleavage, including the presence of canonical RSSs and proper formation of the paired synaptic complex, RAG can occasionally target and cleave non-canonical DNA sequences that resemble RSS motifs, termed cryptic RSSs [24,25]. Off-target cleavage events can result in oncogenic rearrangements [26–30], such as the t(14;18), t(8;14) and t(5;14) chromosomal translocations that drive B cell acute lymphoblastic leukemias/lymphomas (B-ALL) [31–33].

To enable the assembly of a functional primary BCR repertoire while safeguarding genomic integrity, V(D)J recombination is regulated by distinct mechanisms operating at both the pre- and post-cleavage stages. The pre-break phase is primarily defined by the spatiotemporal control of RAG expression and activity (extensively reviewed elsewhere: [34,35]), whereas regulation at the post-cleavage phase is centered on a complex and multilayered network of DNA repair processes that govern end stabilization, processing, and joining.

3. From RAG cleavage to repair commitment

V(D)J recombination promotes diversification of antigen receptor loci at multiple levels, starting with the stochastic assembly of *V*, *D*, and *J* gene segments. The theoretical number of possible recombination events far exceeds the number of B cell progenitors, rendering the potential BCR repertoire virtually limitless (Figs. 1B and 3). Although recent studies have considerably advanced our understanding of the molecular requirements and features of this stochastic selection process, evidence suggests that individual antigen receptor loci adopt distinct mechanisms for chromatin contraction to regulate RAG accessibility to the respective *V, D* and *J* genes [36–43]. An additional layer of diversity arises from the combinatorial pairing of independently rearranged heavy and light chains (Fig. 3). Finally, sequence variability within each recombination event is further increased by the asymmetric processing of coding ends.

While signal ends are blunt and can be directly ligated, coding ends are sealed as hairpins and must be opened before ligation [44] (Fig. 3). The endonuclease ARTEMIS resolves these hairpin structures by introducing asymmetric nicks that typically generate 3′ overhangs of two nucleotides [45–47]. These overhangs are filled in by DNA polymerases, resulting in the formation of palindromic (P) nucleotides, which are short, templated additions at the coding joints. In addition, terminal deoxynucleotidyl transferase (TdT) catalyzes the template-independent addition of random nucleotides (N nucleotides) to the 3′ hydroxyl termini (Fig. 3). Together, these processes generate junctional diversity by introducing unique sequences that are not present in the germline genome [48,49]. It is thus evident that productive rearrangements during BCR diversification inherently deviate from conventional principles of genome fidelity.

Productive V(D)J recombination is ensured *via* the formation of the post-cleavage complex (PCC), in which RAG proteins remain bound to the cleaved DNA ends [15,50]. The PCC not only stabilizes RAG-generated DSBs but also ensures an optimal degree of nucleolytic processing and coordinates their handoff to the NHEJ machinery [15,50]. Repair of DSBs by NHEJ occurs *via* direct ligation or minimal end processing, typically resulting in junctions that are either blunt or contain 1–4 nucleotides of microhomology [51]. Therefore, although NHEJ is considered error-prone in the context of ubiquitous DSB repair, it is specifically harnessed during V(D)J recombination to increase junctional diversity. Disruption of the core NHEJ components KU70/80, DNA ligase IV and XRCC4, nearly abrogates V(D)J recombination [52–58]. The few residual junctions that do form are typically aberrant, often with extensive deletions, increased microhomology usage, or, sporadically, long stretches of non-templated nucleotide insertions [59–62]. These features reflect extensive end-processing and are attributed to the activity of the alternative end-joining (A-EJ) pathway [59–62]. The markedly reduced efficiency of recombination, together with the SCID phenotype observed in these genetic backgrounds, indicate that A-EJ is insufficient to functionally compensate for the loss of canonical NHEJ during V(D)J recombination.

The PCC is not only crucial for ensuring *Ig* gene diversification but also serves to protect the genome from aberrant recombination events, with the ataxia-telangiectasia mutated (ATM) protein long recognized as an essential regulator of PCC stabilization [63]. ATM is a central kinase in the cellular response to DSBs and its deficiency causes ataxia-telangiectasia, a disorder marked by genomic instability, lymphopenia, and a pronounced predisposition to lymphoid malignancies [64]. These cancers often harbor chromosomal translocations involving antigen receptor loci, as loss of ATM function results in aberrant repair events [63]. Multiple additional DSB repair factors, including XLF, PAXX and 53BP1, have later been implicated in the stabilization of the PCC, often with overlapping or redundant functions [15,65–71]. The core NHEJ components, as well as the C-terminal domain of RAG2, also function as negative regulators of chromosomal translocations arising from RAG-induced *Ig* breaks [59–62,72,73].

Therefore, by strictly coordinating the processing and repair of RAG-generated DNA ends, the formation, composition and stabilization of the PCC contribute to the establishment of a locally permissive environment that facilitates imprecise end joining at antigen receptor loci while concurrently limiting the risk of aberrant rearrangements.

4. Secondary antibody diversification and its genomic risks

Once the primary BCR repertoire is established through V(D)J recombination in B cell progenitors, activation of mature B cells in the periphery triggers SHM and CSR [14]. Both reactions are dependent on the B cell specific enzyme activation-induced cytidine deaminase (AID), which converts cytosines to uracils in single-stranded DNA (ssDNA) [74]. The resulting U:G mismatches initiate DNA modification cascades that lead to the introduction of mutations in the variable region of *Igl* and *Igh* loci during SHM, and the formation of DSBs and mutations in the constant region of the *Igh* locus during CSR [74]. These programmed DNA lesions are obligate intermediates of Ig diversification processes, and mutations in *Aicda* (the AID-encoding gene) are responsible for primary antibody immunodeficiencies [74].

Although AID preferentially targets *Ig* genes, it can also induce damage at off-target loci, including *BCL6, PIM1*, *MYC*, and *PAX5* [75–77]. This off-target activity can lead to point mutations, translocations, and clustered hypermutation (kataegis), all of which are associated with the development of mature B cell lymphomas [75–77]. AID-induced point mutations at non-*Ig* loci promote lymphomagenesis by directly altering the coding regions or regulatory elements, primarily promoters, of proto-oncogenes or tumor suppressors such as *BCL6*, *PIM1*, and *PAX5* [78–83]. In parallel, chromosomal translocations involving AID-induced DSBs can juxtapose powerful enhancers, such as those at *Ig* loci, with oncogenes like *MYC*, leading to their aberrant activation [84–86]. Kataegis contributes to lymphomagenesis by introducing dense mutation clusters in regulatory regions or near translocation breakpoints, thus compounding the effects of structural rearrangements. More recently, enhancer hijacking has also been described in the absence of rearrangements. Specifically, AID-induced noncoding mutations at promoters embedded in super-enhancer-rich domains can inactivate promoter function and redirect nearby enhancer activity, contributing to transcriptional dysregulation and B cell lymphoma progression [87]. For example, mutations at the transcription start site of *PAX5* lead to activation of the adjacent gene *ZCCHC7* by surrounding enhancers normally associated with *PAX5* [87]. This mechanism of enhancer retargeting expands the known consequences of noncoding AID activity at non-*Ig* loci beyond traditional promoter mutation and translocation models.

To enable diversification of *Ig* loci while minimizing the risk of

deleterious off-target mutations, B lymphocytes have evolved multi-tiered mechanisms to tightly control AID expression, localization, activity and targeting [88], thus strictly regulating the generation of AID-induced DNA lesions. In parallel, to tolerate the resulting high levels of DNA damage, activated B cells downregulate the expression of key DNA damage checkpoint regulators, such as P53, ATR, and CHEK1, which would otherwise trigger apoptosis [89–91]. These adaptations are critical for mounting an effective antibody response and reflect a trade-off between enabling antibody gene diversification and preserving genome integrity.

5. Mutagenic repair during SHM

The mutation rate in the variable regions of the *Ig* loci reaches approximately 10^{-5}-10^{-3} per base pair per cell division, which is a million-fold higher than the background mutation rate (10^{-10}-10^{-9}) [92,93]. This exceptionally high rate is achieved through the preferential processing of AID-induced U:G mismatches *via* three mutagenic repair branches during SHM [14,92].

Although AID targets the *Ig* loci in G1, the uracils can persist until S phase and serve as templates during DNA replication, resulting in transition mutations (purine-to-purine or pyrimidine-to-pyrimidine) [14,92]. Specifically, replication across the U:G mismatch leads to the incorporation of adenine opposite the uracil, generating a C:G to T:A transition in the newly synthesized DNA strand [14,92] (Fig. 4A). Alternatively, the presence of uracil can be detected by uracil DNA glycosylase 2 (UNG2) or MutSα (heterodimer of MSH2 and MSH6), key DNA repair factors in the base excision repair (BER) and mismatch repair (MMR) pathways, respectively, generating different mutation profiles [14,92,94,95] (Fig. 4A).

Both BER and MMR canonically act to prevent the accumulation of mutations and DNA lesions arising from base modifying agents or errors during DNA replication, respectively [96,97]. BER primarily recognizes and repairs small, non-helix-distorting lesions that affect individual bases, such as those caused by oxidative damage, alkylation and deamination [96]. During canonical BER, UNG2 activity leads to U removal and formation of an abasic site, leaving the phosphate and sugar backbone intact [96]. This step is followed by cleavage of the DNA backbone by apurinic/apyrimidinic (AP) endonuclease 1 (APE1) and fill-in synthesis of the abasic site by the high-fidelity DNA polymerase β (Pol β) (short-patched BER) [96]. Alternatively, BER activity can also lead to re-synthesis of 2–10 nucleotides downstream of the excised uracil through the recruitment of the high-fidelity DNA polymerases Pol β/δ/ε (long-patched BER) [96]. MMR repairs base mismatches, insertions and deletions introduced during DNA replication. MutSα recognizes DNA mismatches and recruits endonucleases that introduce a nick in the DNA strand containing the mismatch [97]. The nick serves as an entry point for exonuclease 1 (EXO1), whose 5' to 3' exonuclease activity removes a stretch of nucleotide encompassing the mismatch [97]. The resulting gap is then refilled by high-fidelity Polδ/ε, restoring the original DNA sequence [97].

While the maintenance of genome integrity relies on high-fidelity BER and MMR, SHM engages non-canonical, error-prone variants of these pathways to skew repair towards a mutagenic outcome, both at AID-targeted cytosines and at nearby nucleotides [14,92]. A key mechanism facilitating mutagenic repair during SHM is the recruitment of translesion synthesis (TLS) DNA polymerases [98]. TLS polymerases differ from their high-fidelity counterparts in that their active sites can accommodate distorted DNA structures [99,100]. This property enables them to replicate across DNA lesions, but at the cost of fidelity, thus considerably increasing the likelihood of introducing mutations [99,100]. During non-canonical BER, REV1, Polη and Polθ fill in the abasic sites generated by UNG2 activity, leading to the formation of both transition and transversion (purine-to-pyrimidine or pyrimidine-to-purine) mutations at AID-targeted C/G pairs [14,98,101–105] (Fig. 4A). Additionally, error-prone MMR fills in the EXO1-generated ssDNA gaps *via* Polη and other TLS polymerases such as Polζ and Polι [14,98,105–107] (Fig. 4A). Moreover, during SHM, the DNA backbone at abasic sites is cleaved not by APE1, but by APE2, an APE1 homolog that has been proposed to mediate TLS polymerases recruitment [108,109] (Figs. 4A and 5).

Non-canonical BER and MMR are responsible not only for the generation of point mutations but also of deletions and insertions, which are observed at much lower frequency during SHM [98,110]. Deletions have recently been proposed to occur *via* a two-step process that is dependent primarily on the non-canonical BER pathway in conjunction with NHEJ-mediated DSB repair [98,110]. On the other hand, the generation of insertions is supported by both non-canonical BER and MMR, likely also *via* DSB intermediates. In this context, nucleotide insertions are thought to result from fill-in synthesis during DSB end joining [98,110].

Non-canonical BER and MMR have long been shown to function epistatically in shaping the full mutational spectrum of SHM [104,111–113]. However, the molecular basis of their coordination was only recently uncovered. FAM72A was recently identified as a crucial player in integrating BER and MMR activities during SHM [114–116]. FAM72A promotes the ubiquitination and subsequent proteasomal degradation of UNG2 specifically during the G1 phase, when AID introduces uracils at the *Ig* loci [116]. Reduced UNG2 levels allow U:G mismatches to persist into S-phase. As FAM72A expression declines in S phase, UNG2 levels increase, allowing uracils in the EXO1-generated ssDNA tracks to be converted into replication-blocking abasic sites that trigger translesional synthesis by Polη [114–116] (Fig. 5).

Hence, through the coordinated modulation of BER and MMR, SHM converts canonical DNA repair into a mutagenic program essential for generating high-affinity antibodies.

6. Generation of DSBs at switch regions during CSR

Naïve B cells express Igs of the IgM or IgD isotype, encoded by the heavy chain constant (C) regions Cμ and Cδ, respectively, *via* an alternative splicing mechanism [14,51]. This isotype expression results from the juxtaposition of the Cμ-Cδ region to the rearranged VDJ exon of the expressed *Igh* allele [14,51]. In mouse B cells, Cμ-Cδ is followed by eight exon sets encoding for the Cx regions of the different isotypes. Each C gene (except Cδ) is preceded by a 5' intronic promoter, an intervening (I) exon, and an intronic switch (S) region [14,51]. The S regions are highly repetitive GC-rich stretches of DNA that extend up to 10–12 kb and differ in their core repeat units [14,51]. CSR occurs *via* a somatic deletional recombination reaction that replaces the Cμ region (donor) with one of the downstream Cx genes (acceptor) [14,51]. Similar to SHM, CSR is initiated in G1 by AID, which targets the S regions preceding the recombining donor-acceptor C pair at the *Igh* locus [14,51]. AID-induced U:G mismatches at the S regions are also processed by BER and MMR; however, in contrast to SHM, this processing results in the generation of DSBs in addition to mutations [14,51] (Fig. 4B).

DSB formation is facilitated by sequence features [117–120], namely G4 structures and R loops, as well as the enrichment of hotspots for AID targeting within the S regions (AGCT motifs) [121]. The high density of these palindromic sequences allows for AID targeting in close proximity on both DNA strands [121]. In contrast to SHM, both APE1 and APE2 contribute to CSR by cleaving the DNA backbone at closely spaced abasic sites on opposite strands, generating staggered single-strand nicks that resolve into DSBs [109,120–122]. Although APE1 is likely responsible for the majority of these nicks due to its high endonuclease catalytic efficiency, APE2 is thought to support DSB formation also through its exonuclease activity [109]. This activity becomes essential when APE1 and APE2 cleave AP sites on opposite strands that are too far apart to form a DSB [123]. In such cases, APE2 can resect 10–12 nucleotides to reach the nick generated by APE1 on the complementary strand [108,109,123] (Fig. 4B). Finally, APE1/APE2-generated nicks can also give rise to DSBs through an MMR-dependent mechanism [124]. When MutSα recognizes uracils on the strand opposite to a BER-generated

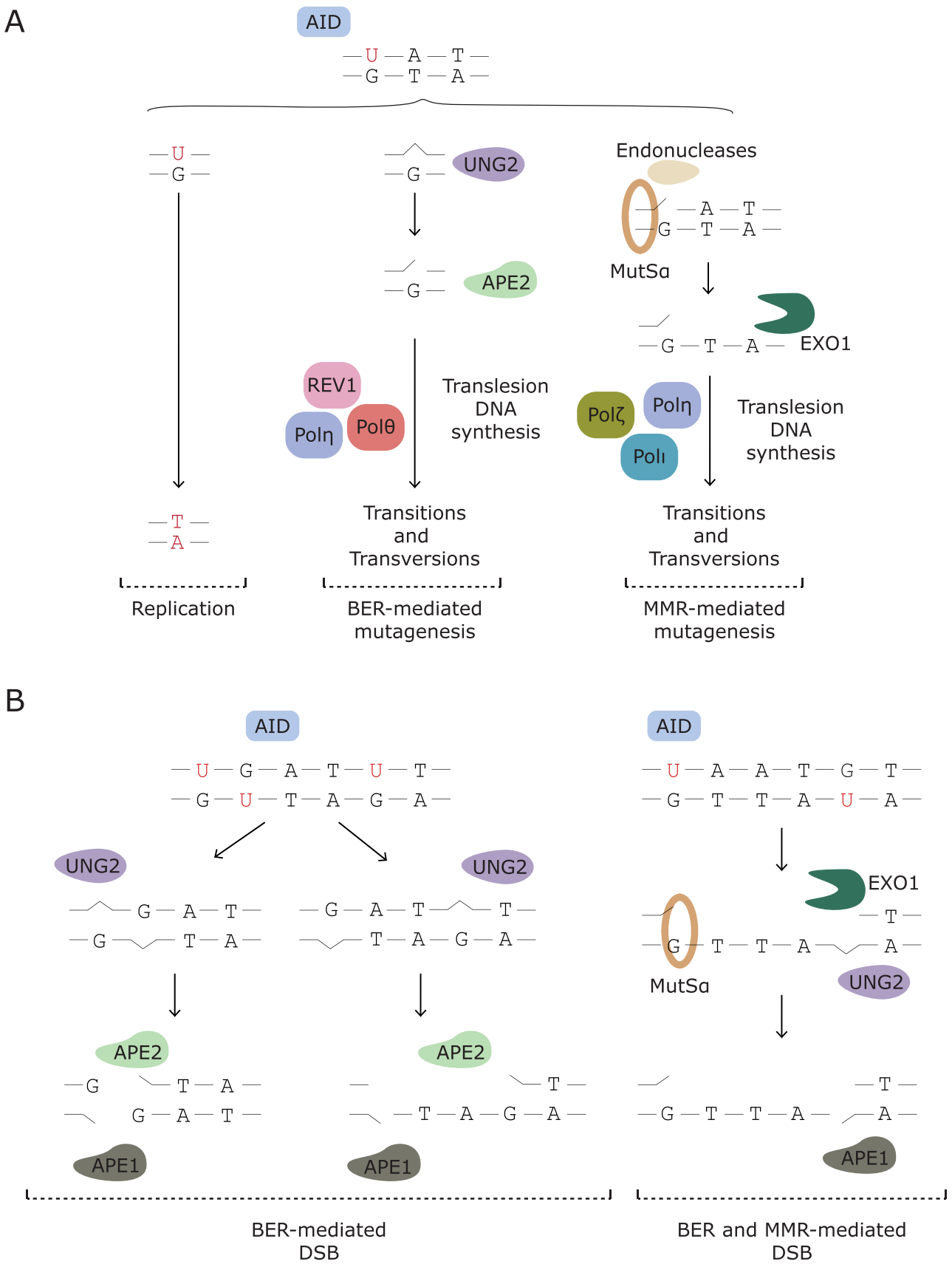

Fig. 4. B cells repurpose ubiquitous DNA repair pathways to drive antibody diversification. A-B) Overview of the mutagenic repair branches that convert AID-induced U:G mismatches into mutations during SHM (A) and DSBs during CSR (B).

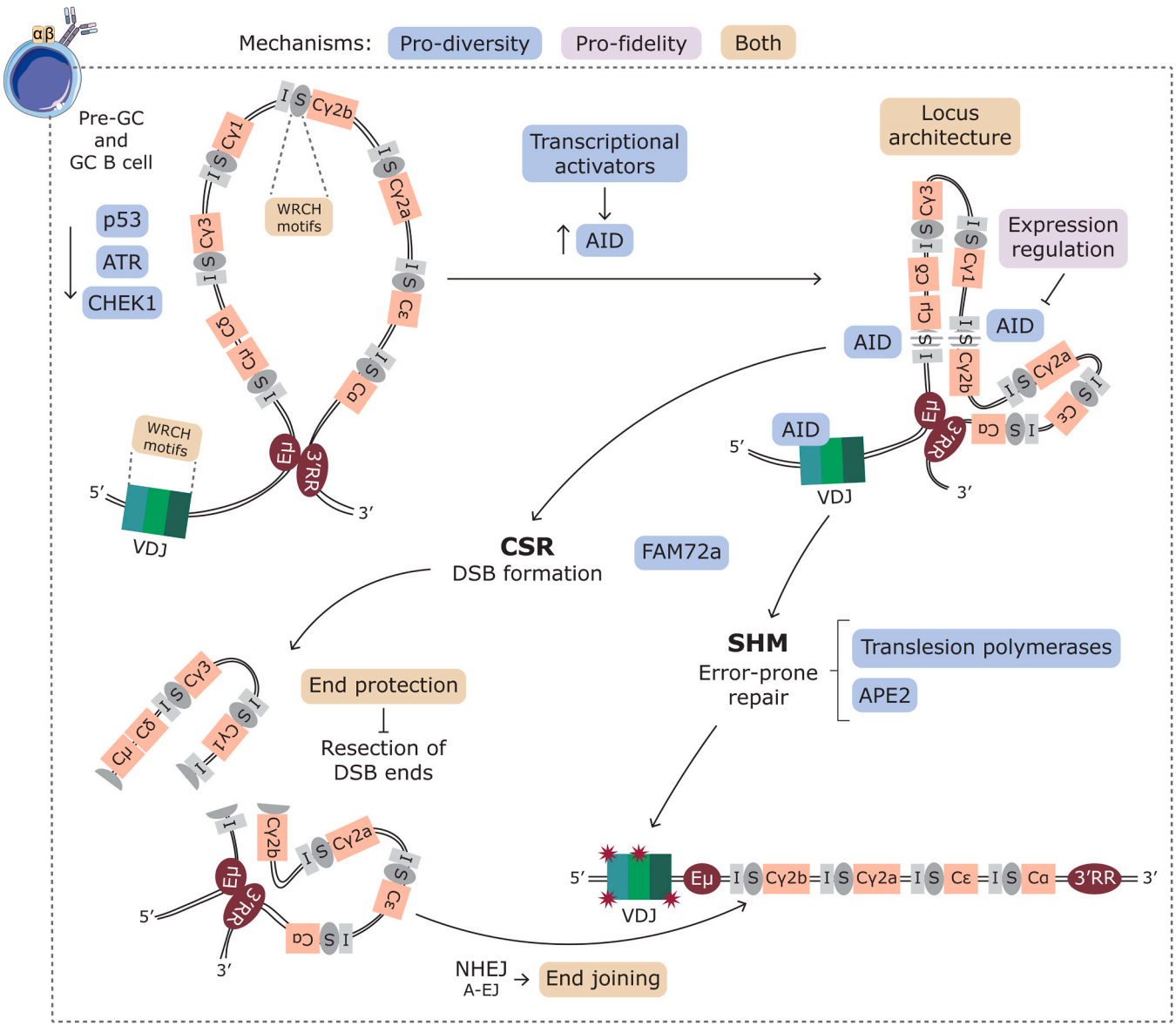

Fig. 5. Mechanisms regulating mutagenic repair during antigen-driven antibody diversification. Schematic overview of key steps of CSR and SHM highlighting the mechanisms that promote antibody diversity (pro-diversity), preserve genome integrity (pro-fidelity), or contribute to both. WRCH: consensus motif for AID targeting hotspots where W = A or T, R = A or G and H = A, C or T.

single strand break (SSB), it triggers EXO1-mediated excision of the uracil-containing strand [124]. A DSB is formed when the excision tract reaches the pre-existing nick on the complementary strand [124] (Fig. 4B). AID hotspots are less frequent outside the S region cores, and as a result, AID targeting in these areas leads to the generation of UNG2- and APE1-derived SSBs that are resolved into mutations [51,125]. This feature is responsible for the footprint of AID-induced mutations that accompany DSB formation during CSR [51,125].

7. Productive *versus* aberrant repair of S region DSBs

Resolution of S region breaks occurs during G1 to early S phase [126], primarily *via* NHEJ [51]. However, deletion of the core NHEJ factors KU70/80, XRCC4 and LIG4 considerably reduces but does not abrogate CSR [127–130]. The residual Sμ-Sx junctions bear a reduced frequency of direct joins and a concomitant increase in both the frequency and length of microhomologies [51,128–131]. These findings provide evidence that AID-induced breaks, unlike RAG-generated DSBs during V(D)J recombination, can also be productively repaired by the A-EJ pathway, albeit with lower efficiency and slower kinetics [51, 128–133]. The delayed repair kinetics likely derive from the additional requirement for limited end resection to expose the microhomologies, as well as the lower abundance of some A-EJ factors [51,128–133].

The regulation of DNA end resection plays a substantially more critical role in determining pathway choice and repair outcome during CSR than in V(D)J recombination [51]. Mechanistically, DNA end resection begins with the recruitment of the multifunctional DNA damage sensor and processing complex MRN (MRE11-RAD50-NSB1) and its co-factor CtIP to DSBs [134,135]. CtIP stimulates the endonuclease activity of the MRE11 subunit, resulting in the formation of a nick on the 5′ DNA strand near the break site. MRE11's processive 3′–5′ exonuclease activity then degrades the same strand from the nick toward the DSB, thus generating ssDNA stretches typically less than 100 nucleotides in length (short-range end resection) [134,135]. The MRE11-generated nicks provide also an entry site for nucleases and cofactors that promote long-range resection, which is mediated by either EXO1, or by BLM and DNA2 [134,135]. EXO1 resects DNA using its 5′–3′ double-stranded DNA exonuclease activity, whereas BLM helicase

unwinds the DNA duplex and the 5′ strand is degraded by the endonuclease activity of DNA2 [134,135].

In contrast to V(D)J recombination, the absence of a defined PCC during CSR leaves AID-induced breaks accessible also to A-EJ, rather than confining them exclusively to the NHEJ pathway [51,127–130]. Additionally, the length and intronic nature of the S regions permit a degree of resection that still allows for productive inter-S joining and enables A-EJ to serve as a backup repair pathway [51,59]. However, the individual S regions are highly repetitive but differ in their core repeat units [136]. As a result, limited resection of DSBs tends to expose short, locally proximal stretches of microhomology that favor intra-S-recombination, a competing, yet unproductive, repair outcome that leads to S region contraction without isotype switching [51, 128–139]. Finally, while limited processing is compatible with productive CSR *via* A-EJ, excessive resection renders them unsuitable for repair by either end-joining pathway [140]. Moreover, resection extending beyond the S regions may reach the adjacent coding C region exons, resulting in loss of BCR expression [140].

Accordingly, the 53BP1-RIF1-Shieldin pathway is a major regulator of AID-induced DSB repair outcomes [14,51]. Specifically, 53BP1 counteracts the formation of extensive 3' resected overhangs by mediating the RIF1-dependent recruitment of its downstream effector Shieldin complex (composed by SHLD1–3 and REV7) [141–148]. Compared to the partial reduction in CSR observed in the absence of a functional NHEJ pathway, defects in any component of the 53BP1-RIF1-Shieldin machinery nearly abrogate CSR [140,148–155]. In addition, a considerable fraction of these B cells loses BCR expression upon activation [140]. Notably, increased levels of intra-S-recombination have been observed in 53BP1-deficient cells, consistent with a shift towards unproductive repair [154–156]. These findings indicate that 53BP1-mediated DSB end protection is essential for productive inter-S joining events leading to CSR by both NHEJ and A-EJ, while safeguarding against the deleterious consequences of extensive resection into the C regions (Fig. 5).

A substantial body of studies has recently uncovered that, protection of ubiquitous DSBs relies on both the inhibition of nucleolytic processing and fill-in DNA synthesis [142–158]. Paradoxically, limited end resection of approximately 50 bp is required to enable protection against extensive processing. The short 3' ssDNA overhangs are necessary for the loading of SHLD2, the ssDNA-binding subunit of the Shieldin complex, which safeguards the ssDNA-dsDNA junction from further resection by blocking access to nucleolytic factors [143,144,146,147–160]. In parallel, SHLD1 recruits the CST complex (CTC1-STN1-TEN1), which in turn brings in its accessory factor polymerase-α (Polα)–primase to restore the minimally resected double-stranded DNA structure [142, 147,157,161–163]. However, since Polα has low processivity and typically synthesizes only short DNA stretches (20 bp), SHLD2 also recruits the structure-specific DNA endonuclease ASTE1 to trim the 3' ssDNA overhang, making it suitable for CST-Polα-mediated fill-in synthesis and end-joining [164,165]. A recent study further expanded the role of CST in DSB end protection by showing that it can directly inhibit the activity of the end resection factors EXO1 and BLM–DNA2, thus supporting a dual function of CST in the regulation of DNA end processing: fill-in DNA synthesis and nuclease inhibition [166].

ASTE1 and Polα-primase-mediated fill-in synthesis are also required for the repair of AID-induced DSBs [164,165]. In addition, DNA polymerase ζ functions epistatically with Shieldin and the CST complex during the repair of S region breaks, further expanding the set of players contributing to DNA end protection by fill-in synthesis in this context [163]. However, SHLD1 is dispensable for Polα recruitment during CSR, presumably because S region DSB ends carry CST recognition sites, allowing for Shieldin-independent recruitment of CST and Polα [157]. These findings suggest that although the regulation of DNA end resection during CSR largely mirrors that of ubiquitous DSBs, the specific mechanistic requirements may differ depending on the nature of the break.

Taken all together, processing of AID breaks is counteracted by both the inhibition of nucleolytic resection and fill-in DNA synthesis, and is a key determinant of whether repair leads to productive, unproductive, or even deleterious outcomes. As such, DSB end protection is essential for both *Igh* diversification and the preservation of genome stability in activated B cells (Fig. 5).

8. Chromatin-driven deletional bias in CSR

Productive class switching results from end joining-mediated deletion of the DNA sequence between the recombining S regions, which places the new isotype C segment directly downstream of the *Igh* variable region [14,51]. Accordingly, repair of CSR breaks exhibits the so-called deletional bias, an orientation-specific recombination preference with a 90:10 ratio of deletions over inversions [167]. This feature stands in contrast to the repair of ubiquitous DNA breaks, which exhibits no such preference and yields a 50:50 ratio of deletions over inversions [14,51,167].

The mechanism underlying the deletional bias has been attributed to the structure and re-organization dynamics of the *Igh* locus upon B cell activation [43]. The locus contains two regulatory elements that are critical for CSR: the intronic enhancer Eμ, located upstream of Sμ, and the 3' regulatory region (3'RR), located at the 3' end of the locus [43, 168,169]. In resting B cells, interactions between Eμ and the 3'RR position all S-C units within a spatially constrained chromatin loop [43, 170] (Fig. 5). Upon antigen encounter, cohesin-mediated loop extrusion generates subloops that synapse the recombining Sμ-Sx regions and align the AID-induced DSBs within these regions in a configuration that specifically favors deletional repair [43,170] (Fig. 5). Several studies indicate that *Igh* locus conformation and dynamics are influenced by DSB end protection and repair factors, including 53BP1 and ERCC6L2, as their deletion reduces or abrogates the CSR deletional bias [167, 171–173]. However, how these proteins enforce the bias and whether they do so by influencing the locus architecture remains unclear [174].

In summary, antibody diversification by CSR is also supported by the establishment and maintenance of higher-order chromatin structures [43]. Since deletion of architectural *Igh* elements leads to aberrant ligation of CSR breaks to ectopic sequences [175,176], these same structural features also appear to contribute to the preservation of genome integrity by limiting the generation of AID-mediated translocations [175–177].

9. Concluding remarks

B lymphocytes are unique in their ability to sustain programmed DNA damage across three distinct antibody gene diversification reactions, enabling the generation of a broad and protective humoral immune repertoire [178]. While the spatiotemporal regulation of RAG and AID activity has been extensively studied, many questions remain about the molecular logic that governs how DNA repair pathways are selectively engaged and functionally redirected during these processes.

In the context of V(D)J recombination, the precise mechanisms by which RAG-induced DNA ends are directed toward resolution by the NHEJ machinery remain unclear. Specifically, despite decades since ATM was first implicated in the stabilization of the PCC [63], and the expanding list of additional contributing factors [65–68,70,179,180], how these components cooperate to maintain PCC integrity and coordinate DNA end processing remains poorly defined [174].

In activated B cells, a still largely unresolved question is how DNA repair pathways that are ubiquitously dedicated to preserving genome integrity are repurposed to promote targeted mutagenesis at *Ig* loci. This functional duality is especially demanding given that these pathways must operate in a mutagenic mode at *Ig* genes while simultaneously maintaining high-fidelity function throughout the rest of the genome [14,51]. The challenge is further amplified by the fact that programmed AID-induced lesions occur in parallel with additional genotoxic stressors

intrinsic to antigen-driven activation, such as rapid cell proliferation and metabolic reprogramming [181–183].

Emerging evidence suggests that activated B cells employ safeguarding mechanisms to mitigate excessive mutagenesis even at the *Ig* loci themselves. During SHM, the primase-polymerase PrimPol is known to restrain excessive REV1-driven mutagenesis [184]. Similarly, the DNA crosslinking factor HMCES was recently reported to limit the formation of deletions during SHM by shielding UNG2-generated abasic sites from APE2-mediated nicking, which would otherwise result in DSBs [185]. These findings indicate that, while non-canonical DNA repair pathways are harnessed to drive diversification, B cells also deploy counterbalancing mechanisms to locally restrict their mutagenic potential, further underscoring the importance of maintaining a balance between error-prone and error-free repair.

In the context of CSR, recent studies have highlighted the importance of RNA processing in preserving the balance between *Igh* diversification and genome stability. Noncoding transcription at S regions, known as germline transcription (GLT), facilitates AID targeting *via* R loop formation, but resolution of these structures through RNA degradation is essential for productive repair [14,186]. Disruption of GLT turnover, *via* loss of the RNA exosome catalytic subunit DIS3 or GLT methylation by methyltransferase-like 3 (METTL3), leads to R-loop accumulation, impaired CSR, and increased *Igh* translocations [187,188]. Notably, DIS3 deficiency also disrupts the coordination of AID-induced nicks on both DNA strands, resulting in strand-biased processing and a repair shift marked by increased microhomology at S–S junctions [187]. Similarly, loss of the RNA-binding protein HNRNPU, which facilitates R-loop resolution during CSR, has been linked to reduced NHEJ usage and increased microhomology, consistent with a shift in repair pathway engagement [189]. These findings also illustrate how the mechanism and coordination of AID-induced DSB formation at *Igh* can directly influence repair pathway choice and recombination outcome.

Although AID-induced mutations at non-*Ig* loci are less frequent than those at *Ig* genes, the outcome of their repair is influenced by the interplay of multiple pathways and shaped by gene-specific sequence and chromatin context. Studies using $Ung^{-/-}Msh2^{-/-}$ mice have shown that the BER and MMR pathways efficiently suppress most off-target mutations, although their effectiveness depends on gene identity, sequence context, and possibly mutational load [76]. In cases where repair fails, translocations can arise, often involving actively transcribed non-*Ig* genes that reside in close nuclear proximity to *Igh* or within 3D enhancer hubs [118,190,191]. Additionally, AID targeting at non-*Ig* loci may depend on distinct sequence motifs or chromatin features not shared with *Ig*-associated breaks [192]. While these studies reveal several factors influencing off-target repair, we still lack a predictive framework to determine which AID-induced lesions are faithfully resolved and which progress toward transformation.

In conclusion, how the equilibrium between permissive mutagenesis and genome preservation is maintained remains incompletely defined. Deciphering the molecular logic and regulatory architecture that govern DNA repair dynamics during antibody diversification will not only deepen our understanding of B cell biology, but may also uncover broader mechanisms of genome protection.

CRediT authorship contribution statement

Michela Di Virgilio: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Eleni Kabrani:** Writing – review & editing, Writing – original draft, Conceptualization. **Maria Berruezo-Llacuna:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization.

Declaration of Competing Interest

The authors declare no competing interests.

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