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Review

Charting a DNA Repair Roadmap for Immunoglobulin Class Switch Recombination

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Immunoglobulin (Ig) class switch recombination (CSR) is the process occurring in mature B cells that diversifies the effector component of antibody responses. CSR is initiated by the activity of the B cell-specific enzyme activation-induced cytidine deaminase (AID), which leads to the formation of programmed DNA double-strand breaks (DSBs) at the Ig heavy chain (*Igh*) locus. Mature B cells use a multilayered and complex regulatory framework to ensure that AID-induced DNA breaks are channeled into productive repair reactions leading to CSR, and to avoid aberrant repair events causing lymphomagenic chromosomal translocations. Here, we review the DNA repair pathways acting on AID-induced DSBs and their functional interplay, with a particular focus on the latest developments in their molecular composition and mechanistic regulation.

Highlights

Mature B cells rely on a multilayered regulatory framework to ensure that S region DSBs are preferentially channeled into the NHEJ pathway.

The structure of AID-induced breaks influences both the DSB end-processing mode and end-joining pathway choice.

Both the SSA factor RAD52 and the A-EJ protein HMCES contribute strand pairing activities during repair of S region DSBs.

Repair of AID-initiated breaks is influenced by *Igh* locus-specific organizational features that ensure productive end-joining events leading to CSR. 53BP1 contributes both structural and resection modulatory roles to the regulation of CSR repair outcomes.

Shieldin and CST complexes are 53BP1 and Rif1 downstream effectors, and actively counteract the processing of S region DSBs into ssDNA by combining inhibition of DNA end resection and limited fill-in synthesis of resected tracks.

Breaking the B Cell Genome to Diversify Antibody Responses

Our immune system is able to generate a diverse repertoire of **antibodies** (or Igs, see Glossary) that can collectively recognize and efficiently dispose of an impressive number of pathogens. Antibodies are produced by terminally differentiated B lymphocytes known as plasma cells, and can use different routes to eliminate pathogens. These alternative effector functions are specified by the antibody isotypes (or classes) [1]. A mature B lymphocyte diversifies the class of antibody it expresses *via* the process of Ig CSR [2,3]. CSR replaces the constant portion of the IgM heavy chain with one of the alternative isotypes (IgG, IgA, or IgE), thus changing the antibody effector function without altering its specificity [1]. At the molecular level, CSR is a somatic recombination reaction that occurs *via* the programmed formation and repair of **DNA double-strand breaks** (DSBs) within the Ig heavy chain (*Igh*) locus (Figure 1) [2,3]. The temporary disruption of genome integrity that CSR entails places the process at the crossroads between the establishment of protective immunity, and the need to maintain genome stability. The inability to introduce or repair these programmed DSBs is responsible for primary antibody deficiencies (CSR immunodeficiencies) [4,5]. Conversely, CSR-dependent DSBs can be the substrate of aberrant repair reactions leading to **chromosomal translocations**, which are a hallmark of several mature B cell lymphomas [6]. Therefore, isotype diversification is a fundamental aspect of mature B lymphocyte physiology with important implications in health and disease. In view of the recent COVID-19 pandemic, which has brought to worldwide attention the crucial health and societal roles played by protective immunity, a comprehensive understanding of the processes contributing to **humoral responses** is particularly timely. In this review, we describe the functional interplay between DNA repair pathways operating on CSR DSBs, and we highlight the recent developments on the molecular composition and mechanistic aspects of their regulation. Since CSR has been extensively investigated in mice, we refer primarily to the mouse *Igh* locus and to the molecular details of the reaction obtained in this model system, with reference to other organisms where appropriate.

AID-Induced DSB Formation

The productive *Igh* allele of mature B cells comprises a **rearranged VDJ exon**, and eight exon sets encoding for the constant (C) regions (referred to as C genes) of the different isotypes [7]

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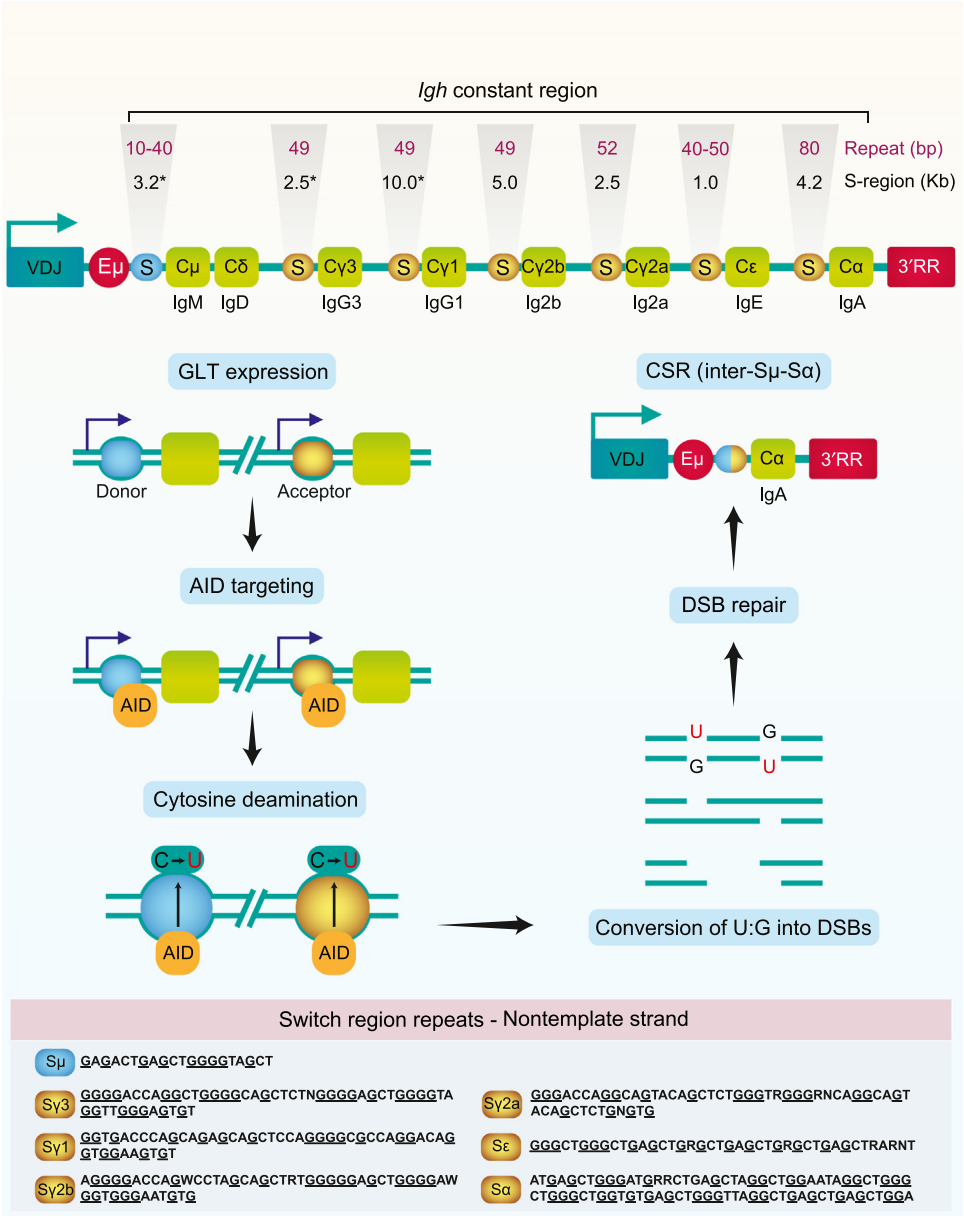

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Figure 1. CSR is a Multistep Deletional DNA Recombination Reaction. The mouse *Igh* locus is located on chromosome 12 (chromosome 14 in humans), and in a mature B cell it comprises a rearranged VDJ exon and eight constant exon sets (C_x genes), which encode for the different immunoglobulin isotypes. Each C gene, apart from Cδ, is preceded by an S region. The approximate length of each S region (the asterisk indicates that the S region length varies in different mice strains) and corresponding repeat units are indicated on top of the *Igh* locus schematic, whereas the sequences of the S region repeat units (nontemplate strand) are shown at the bottom [2,8]. G bases are underlined to highlight the repeat sequences' G-rich content. In resting B cells, the VDJ exon is transcribed with the first C region (Cμ) and the cell expresses an antibody of the IgM class. B cell activation induces AID expression and its GLT-dependent targeting to the donor Sμ and one of the downstream acceptor S regions (Sα in the figure example). AID deaminates C residues to U in single-stranded DNA stretches within the S regions, and the resulting U:G mismatches are converted into nicks and gaps by the base excision repair and mismatch repair pathways. Relatively close nicks and gaps resolve into

(Figure legend continued at the bottom of the next page.)

Glossary

Antibodies (also known as Igs): proteins comprising two heavy and two light chains arranged to create a variable (V) domain, which binds the antigen and defines the antibody specificity, and a constant (C) domain, which specifies the effector function.

Base excision repair: DNA repair pathway that corrects small base lesions. BER first excises the damaged base via a DNA glycosylase, and then nicks the DNA at the resulting abasic site. The nick is further processed to allow replacement of the single nucleotide (short-patch BER) or of a nucleotide stretch (long-patch BER).

Chromosomal translocations: genome rearrangements generated when a broken portion of a chromosome reattaches to a different chromosome.

CRISPR/Cas9-based gene editing: gene-editing technology that adapted the CRISPR/Cas9 genome editing system from bacteria to target DNA in a programmable manner and induce DSBs or nicks at specifically defined genomic locations.

Cytokines: proteins released by cells involved in autocrine, paracrine, and endocrine signaling to affect functions of own, proximal, or distal cells, respectively. In *in vitro* B cell cultures, different cytokine combinations stimulate CSR to different isotypes.

Direct joins: repair junctions without microhomologies or insertions.

DNA damage response: complex network of cellular pathways that coordinate cell cycle progression with the detection, signaling, and repair of DNA damage.

DNA double-strand breaks: highly toxic DNA lesions generated when both strands of the DNA duplex are severed simultaneously.

Exo- or endo-nucleolytic processing: DNA processing mediated by enzymatic activities that either remove one nucleotide at a time from the DNA ends (exonucleases) or introduce nicks/DSBs by hydrolyzing internal phosphodiester bonds (endonucleases).

Gap: stretch of ssDNA within a dsDNA molecule.

G1/S DNA damage checkpoint: temporary arrest in cell cycle progression at the G1/S transition induced in response to DNA damage occurring in G1.

(Figure 1). Except for Cδ, each constant gene is preceded by a constitutively expressed (Cμ region) or **cytokine**-inducible (downstream C regions) 5′ intronic promoter, an intervening (I) exon, and an intronic switch (S) region. S regions are highly repetitive stretches of DNA that extend up to 10–12 kb (Figure 1) [8]. They bear different repeat units of 10–80 bp in length and are G-rich on the nontemplate strand [2]. Cδ is cotranscribed with Cμ, and IgD expression results from alternative splicing of a pre-mRNA transcript composed of the VDJ exon and both C genes. However, a noncanonical S-like region 5′ to Cδ has been identified, and rare Cμ to Cδ CSR events have been reported in specific subsets of B cells in both mice and humans [9–12].

Following B cell activation, DSBs are introduced into the donor Sμ and one of the downstream S acceptor regions as result of the activity of the B cell-specific enzyme AID [13]. AID deaminates cytosine residues to uracil in single-stranded DNA (ssDNA) stretches that are exposed by noncoding transcription (germline transcription; GLT) across the recombining S–C regions [7]. Transcription of a particular acceptor S region promoter results in class switching from IgM to the corresponding antibody isotype. The U:G mispairs are further processed by the **base excision repair** (BER) and **mismatch repair** (MMR) pathways, which results in the generation of multiple DSBs per S region [14].

AID initiates also **somatic hypermutation** (SHM), the additional antibody diversification reaction occurring in mature B cells [14]. During SHM, AID deaminates cytosines at the variable regions of both *Igh* and Ig light chain (*Igl*) loci. The resulting mismatches are converted into single point mutations that have the potential to increase the binding affinity of the antibody molecule towards its cognate antigen [14]. Because of its role in initiating both CSR and SHM, AID activity is crucial for humoral immunity; however, it is also a source of genome instability. Although *Ig* loci represent the preferential targets, AID introduces DNA lesions also in non-*Ig* genes throughout the genome (off-targets), many of which are translocation partners in mature B cell lymphomas [6].

DSB Repair Pathways Operating on CSR Breaks

Mature B cells hijack the ubiquitous **DNA damage response** pathways to sense, transduce, and repair CSR breaks [15]. DSBs are detected by the MRE11–RAD50–NBS1 (MRN) complex, which activates ataxia telangiectasia-mutated kinase (ATM). ATM is the main kinase in the DSB signaling cascade, and once activated, it phosphorylates a plethora of factors, including the histone variant H2AX. Phosphorylation of H2AX (γH2AX) is a key intermediate step in the cascade as it promotes the assembly of DSB signaling and repair factors over large chromatin regions surrounding the break site. Accordingly, deficiencies for ATM, the MRN complex, and H2AX result in impaired CSR [15].

Mammalian cells have evolved four mechanistically distinct pathways to repair DSBs: nonhomologous end joining (NHEJ), alternative end joining (A-EJ), single-strand annealing (SSA), and homologous recombination (HR) (Figure 2). These pathways operate with different kinetics, are mediated by diverse sets of factors, and differ in the extent of sequence homology used in the repair process [16,17]. Furthermore, they are regulated in a cell-cycle-dependent manner with HR and SSA occurring predominantly in S and G2 phases, whereas NHEJ and A-EJ are active throughout G1/S/G2 phases [16]. Since the formation of AID-initiated DNA damage occurs in early

Humoral responses (also known as antibody-mediated responses): immune responses that are mediated by the capability of antibodies to efficiently recognize and dispose of pathogens that are freely circulating or outside infected cells.
Microhomology: DNA sequence at a repair junction that can be assigned to either of the two DNA ends being ligated.
Mismatch repair: DNA repair pathway that corrects mispaired DNA bases. MMR first introduces a nick in the vicinity of the mismatch and then processes it into a patch of ssDNA by removal of nucleotides across the mismatched base.
Nicks: discontinuity affecting only one strand of the DNA molecule and caused by disruption of the phosphodiester bond between adjacent nucleotides.
Preassembled heavy and light chain variable region exons: knocked-in productive heavy and light chain variable region exons. This strategy reconstitutes a monoclonal mature B cell compartment, thus bypassing the dependency of V(D)J recombination and lymphocyte development on NHEJ.
Rearranged VDJ exon: exon generated by the process of V(D)J recombination in developing lymphocytes, and encoding for the antigen-binding (variable) portion of the antibody molecule.
Somatic hypermutation: secondary antibody diversification reaction that introduces mutations at the rearranged V(D)J regions of *Igh* and *Igl* loci with a high frequency, thus enabling the generation of high-affinity antibodies.

DSBs of different structures. Inter-Sμ/S_x region DSB repair results in the formation of a hybrid $S\mu/_X$ junction ($S\mu/\alpha$ in the figure example) and deletion of the intervening DNA sequence. As a consequence, the rearranged VDJ exon is transcribed together with a new constant region (Cα), which encodes for the switched isotype (IgA). Abbreviations: 3′RR, 3′ regulatory region; AID, activation-induced cytidine deaminase; C, cytosine; CSR, class switch recombination; DSB, double-strand break; G, guanine; GLT, germline transcription; U, uracil.

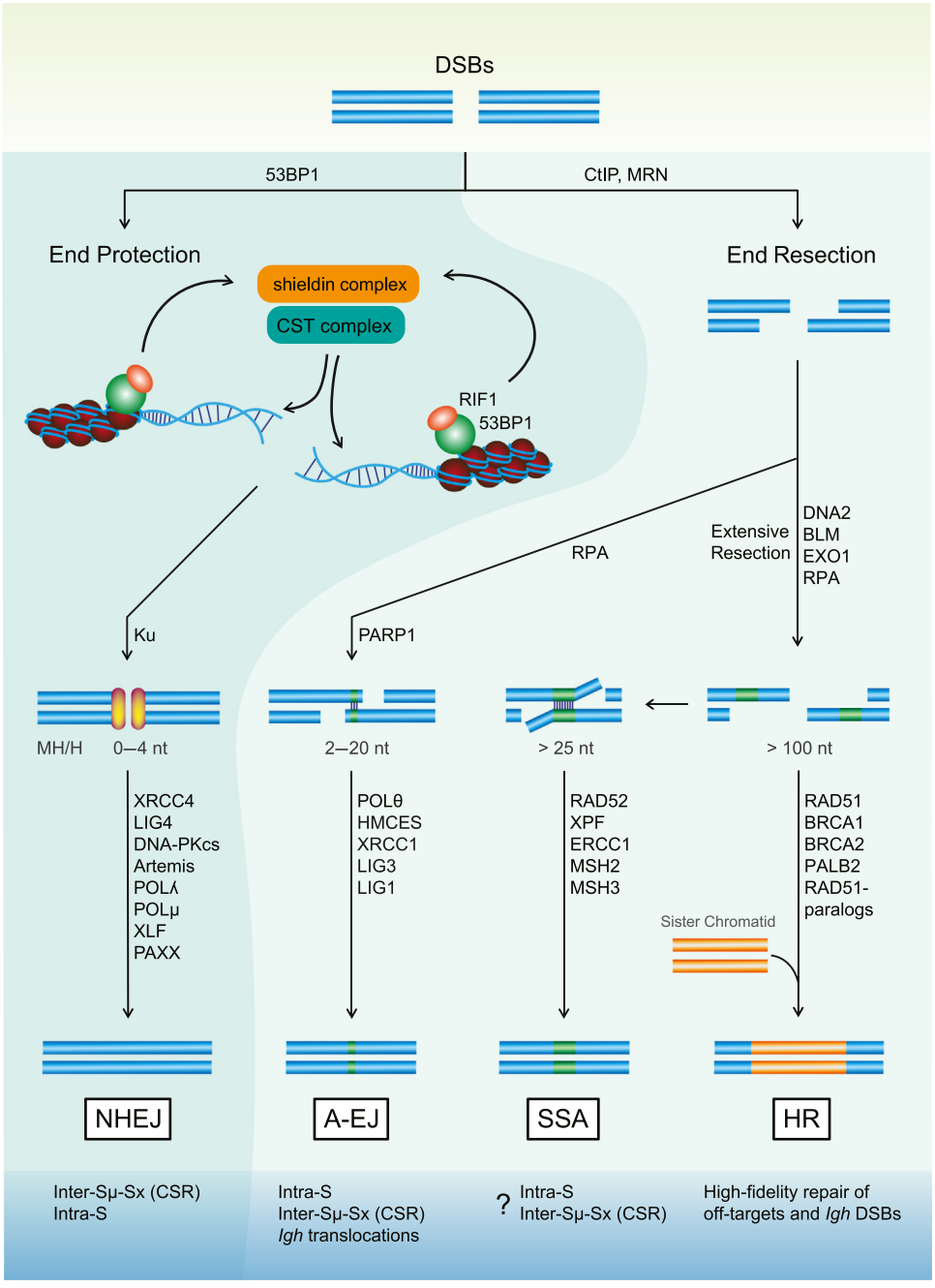

Figure 2. DSB Repair Pathways and Their Contribution to the Resolution of AID-Induced Breaks. Schematic representation of DSB repair pathways operating in mammalian cells and respective key components. The extent of MH/H characteristic of each pathway is indicated. Nonhomologous end joining (NHEJ) repairs DSBs after no or limited DNA end processing, and relies on 53BP1–RIF1–Shieldin–CST-dependent protection of DSB ends. NHEJ is mediated by the core components Ku, XRCC4, and LIG4 with the intervention of several DNA end processing and accessory factors. A-EJ, SSA, and HR are all initiated by 5′–3′ resection of DSB ends. The resulting 3′ single-stranded (ss)DNA strands are coated with and stabilized by RPA. During A-EJ, terminal microhomology pairing by POLθ facilitates ligation by XRCC1–LIG3 or

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G1 phase and CSR is completed by late G1/early S phase [18–21], NHEJ and A-EJ are the major pathways involved in repair of CSR breaks. The term **microhomology**-mediated end joining (MMEJ) is sometimes used as a synonym of A-EJ. However, the interchangeable use of the two terms might be misleading since NHEJ also generates junctions with microhomologies. In this review, we refer to NHEJ and A-EJ as genetically distinct pathways, which can both use microhomologies during repair to anneal the ends and enable ligation.

NHEJ versus A-EJ

NHEJ is the major DSB repair pathway in mammalian cells [22,23]. NHEJ repairs the broken ends via a direct ligation mechanism that requires the KU70/KU80 (Ku) heterodimer and the DNA ligase IV complex, which comprises DNA ligase IV (LIG4) and its cofactor X-ray repair cross-complementing (XRCC)4 (Box 1 and Figure 2). Ku, LIG4, and XRCC4 are evolutionary conserved in their role in NHEJ and are considered core NHEJ components. Although NHEJ of clean ends (blunt ends and compatible overhangs) can occur by direct ligation, end joining of complex ends (incompatible overhangs or ends bearing chemical modifications) requires the intervention of additional enzymes and DNA end processing factors to render them compatible for ligation [22,23]. Modification of DSB ends in the context of NHEJ occurs primarily *via* **exo- or endo-nucleolytic processing** of 5′ or 3′ overhangs and results in the exposure of short stretches of homology typically up to four nucleotides [22,23]. These microhomologies greatly facilitate end joining because terminal base pairing allows for the formation of relatively stable reaction intermediates. The limited nucleolytic processing and **Gap**-filling synthesis necessary to generate ligatable ends are responsible for the small deletions and insertions characteristic of NHEJ junctions.

The composition and mechanism of the A-EJ pathway are less defined [22,24,25] (Box 2 and Figure 2). A-EJ shares some aspects of the more homology-based types of repair (HR and SSA). A-EJ, HR, and SSA are all initiated by nucleolytic degradation of the 5′ strands to generate 3′ ssDNA stretches, in a process referred to as 5′–3′ DNA end resection (Box 3). Resection during A-EJ exposes stretches of sequence complementarity ranging from two to 20 nucleotides on the two strands [22,24,25]. These stretches allow for terminal strand pairing, and lead to junctions characterized by an increased usage and length of microhomologies compared to the ones generated by NHEJ. It should be noted that although the A-EJ pathway is biased towards the use of microhomologies, both in frequency and in length, it can also generate junctions bearing no homology stretches.

Interplay between NHEJ and A-EJ at the Switch Regions

S region junctions in mouse B cells display a 30–50% frequency of **direct joins** [26–29]. The remaining junctions mostly bear 1–4-bp microhomologies and only few are found with microhomology stretches longer than 4 bp [26–29]. Deletion of core NHEJ components in mice carrying **preassembled heavy and light chain variable region exons**, or conditional

LIG1. SSA proceeds via RAD52-dependent pairing of the exposed microhomologies. The noncomplementary 3′ ssDNA flaps are removed by the nucleotide excision repair complex XPF–ERCC1 and the mismatch repair complex MSH2–MSH3, and DNA ligation ultimately completes the process [22,70]. During HR, RPA is replaced by RAD51 on the resected 3′ ssDNA via intervention of the BRCA2/PALB2 complex, which, in concert with the RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3), results in the formation of the nucleoprotein filaments responsible for homologous pairing and strand invasion of the sister chromatid [114]. Only HR factors participating in the early steps of HR are indicated. The contribution of each pathway to the repair of AID-induced breaks is indicated at the bottom. Abbreviations: A-EJ, alternative end joining; AID, activation-induced cytidine deaminase; BLM, Bloom syndrome RecQ-like helicase; CSR, class switch recombination; CST, CTC1–STN1–TEN1 complex; DNA2, DNA replication helicase/nuclease 2; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; EXO1, exonuclease 1; HMCES, 5hmC binding embryonic stem cell-specific protein; HR, homologous recombination; LIG, DNA ligase; MH/H, microhomology/homology; PARP1, poly(ADP-ribose) polymerase 1; PAXX, paralog of XRCC4 and XLF; RPA, replication protein A; SSA, single-strand annealing; XLF, XRCC4-like factor; XRCC, X-ray repair cross-complementing.

Box 1. DSB Repair by NHEJ

NHEJ repair of DSBs initiates with the binding of Ku to the DNA ends [22,23]. Ku is a particularly abundant cellular complex and has a high affinity for DNA ends. As a consequence, Ku is usually the first factor to bind a broken DNA end. During NHEJ repair of blunt DSB ends lacking microhomology, Ku promotes the association of the XRCC4–LIG4 complex to the DNA ends to catalyze the ligation reaction on either strand of the DNA duplex [22,23]. When processing of DNA ends is a prerequisite for repair by NHEJ, DNA-PKcs and the nuclease Artemis are recruited to the Ku-bound DNA ends [22,23]. Artemis possesses a DNA-PKcs-dependent endonuclease activity that acts on both 5′ and 3′ DNA overhangs of duplex DNA at ssDNA to double-stranded DNA (dsDNA) transitions and on DNA hairpins [102]. In addition, it displays DNA-PKcs-independent 5′ exonuclease activity on ssDNA. Artemis can also act on blunt DNA ends able to transiently open into a pseudo-Y structure, and resect into the duplex to expose microhomologies, which explains why repair of some blunt ends occurs *via* loss of nucleotides and microhomology-mediated annealing. Although other nucleases may contribute to the processing of DNA ends, Artemis appears to be the key nuclease involved in the majority of NHEJ events.

In addition to nucleolytic degradation, DNA ends can be modified also by the intervention of DNA polymerases, with the Pol X family members POLλ and POLμ being the primary polymerases involved in NHEJ [22,23]. POLλ incorporates nucleotides predominantly in a template-dependent manner, and this activity contributes to fill-in synthesis of gaps generated by annealing of long ssDNA ends with terminal microhomologies [103]. POLμ facilitates ligation of mismatched 3′ overhangs by adding nucleotides to the overhang ends in both template-dependent and -independent manner, and eventually promotes the formation of regions of microhomology for DNA end annealing and ligation [104]. Additional accessory proteins, such as tyrosyl DNA phosphodiesterase (TDP)1, polynucleotide kinase (PNK), and aprataxin might participate to NHEJ repair of a minor fraction of ends that cannot be processed by the action of the factors described in the previous text [22,23]. Furthermore, the XRCC4-like factor (XLF; also known as Cernunnos) and paralog of XRCC4 and XLF (PAXX) have been suggested to stimulate the activity of the XRCC4–LIG4 complex on incompatible DNA ends by stabilizing their juxtaposition in the absence of the stability conferred by strand annealing [22,23]. Finally, the modulator of retrovirus infection (MRI; also known as CYREN) was recently identified as an adaptor protein able to promote NHEJ in G1 by facilitating the recruitment and/or retention of DNA damage response and NHEJ factors to DSBs [39,105,106]. Both PAXX and MRI have partially overlapping functions with XLF during NHEJ [39–42].

inactivation in mature B cells showed that deficiency for Ku, XRCC4, and LIG4 considerably impairs CSR and results in frequent *Igh* locus DSBs [28–31]. DNA-dependent protein kinase catalytic subunit (DNA-PKcs)-deficiency yields variable degrees of reduction in CSR efficiency [32–34], whereas B cells lacking Artemis or XRCC4-like factor (XLF) display near physiological levels of class switching or only moderate impairment, respectively [35–37]. Nevertheless, they all exhibit AID-dependent *Igh* locus breaks [37,38]. MRI deficiency modestly affects CSR whereas paralog of XRCC4 and XLF (PAXX) is dispensable [39–42]. These observations indicate that AID-induced DSBs are joined largely by NHEJ, and that DNA processing is either not a prerequisite for repair in the majority of events, or it involves multiple, and in part yet-to-be-defined, factors that can act redundantly on AID-induced DSBs.

Box 2. DSB Repair by A-EJ

During repair by A-EJ, Poly(ADP-ribose) polymerase 1 (PARP1) competes with Ku for binding to DSB ends, and rapidly recruits the MRN complex [22,24,25]. MRN and its cofactor CtBP-interacting protein (CtIP; also known as RBBP8) process DSB ends by limited resection, which uncover short stretches of homology (Box 3). Both PARP1 and the MRN complex have been suggested to contribute to DNA end-bridging and strand alignment via microhomologies during A-EJ [25]. More recently, the A-family DNA polymerase member POLθ has been identified as a key component of A-EJ [107,108]. POLθ is a low-fidelity DNA polymerase helicase that appears to contribute multiple functions during repair by A-EJ [24,55]: it displaces RPA from the ssDNA regions while limiting the loading of RAD51 recombinase on resected DNA, thus inhibiting HR; it contributes to microhomology searching, alignment and end tethering; and it participates to error-prone fill-in synthesis of gaps. In addition to template-directed synthesis, POLθ has robust terminal transferase activity, and both activities contribute to generate nucleotide insertions at repair junctions [109]. It is likely that the pathway comprises other DNA polymerases, as well as additional factors and functionally redundant nucleases that participate to the formation of the 3′ ssDNA fragments and removal of the short non-complementary 3′-flaps generated by microhomology pairing. The resolution of DNA repair by A-EJ occurs by ligation of now-compatible ends on both strands. Vertebrates have three ATP-dependent ligases (LIG1, LIG3, and LIG4), with LIG4 acting exclusively during NHEJ. Both LIG1 and LIG3 can provide the ligase activity in the A-EJ pathway, although LIG3 has a more central role than LIG1 [24,25]. Nuclear LIG3 forms a stable complex with the X-ray complementation factor 1 (XRCC1) [110], and the XRCC1–LIG3 complex appears to be recruited to DSBs during A-EJ via interaction with the MRN complex [111,112]. LIG1 has been suggested to contribute to some A-EJ repair events that do not apparently rely on microhomologies [113].

Despite the major CSR reduction observed in B cells lacking NHEJ core components, CSR still occurs at considerable levels in these cells [28–30]. Furthermore, CSR junctions from B cells deficient for XRCC4, LIG4, Ku, XLF, and Artemis exhibit an increased usage of microhomologies, albeit with varying degrees of phenotypic difference compared to wild-type cells [28–30,35,37]. A reduced frequency of direct joins and shift towards long microhomologies were observed also in CSR junctions from patients bearing mutations affecting LIG4, DNA-PKcs, Artemis, and XLF/Cernunnos [43–46]. Altogether, and taking into consideration also the accumulation of *Igh* locus breaks in cells lacking several NHEJ factors [28,37,38], these observations indicate that S region DSBs are accessible to both end joining pathways and can be repaired also by A-EJ, although not as efficiently as by NHEJ.

A-EJ repair of AID-induced DSBs is dependent on poly(ADP-ribose) polymerase (PARP)1 and CtBP-interacting protein (CtIP) [47,48]. Abrogation of PARP1 skews CSR junctions towards reduced use and length of microhomologies, but yields no reduction in CSR efficiency [47]. Analogously, downregulation of CtIP in the mouse B cell lymphoma line CH12 mildly reduces CSR and biases end joining towards direct joins and shorter microhomologies [48], whereas CtIP-deficient primary B cells exhibit no-to-moderate reduction in CSR and junction profiles comparable to wild-type cells [49,50]. Loss of MRE11 results in a profound CSR defect due to the upstream role of the MRN complex in ATM activation during DSB signaling; however, specific inactivation of its nuclease activities causes only moderately reduced CSR efficiency without significantly altering the junction profiles [51]. Altogether, these findings suggest that A-EJ may account for a limited portion of CSR repair events in the presence of an active NHEJ pathway.

POLθ ablation does not affect CSR efficiency or microhomology usage [52–54]. The only CSR-related phenotype observed in POLθ-null B cells is the loss of the small fraction of end joining

Box 3. 5′-3′ DNA End Resection

The initiation of HR requires extensive 5′ to 3′ resection of DNA ends to generate the 3′ ssDNA filaments that are competent for strand invasion of the homologous sequence [114]. These long ssDNA stretches can extend for a few thousand base pairs from both DNA ends and are the result of a two-step mechanism that involves the intervention of several factors [115] (Figure I). The initial, more limited, trimming phase (short-range resection) is mediated by the activity of the MRN complex and CtIP [115,116]. MRE11 possesses 3′ to 5′ dsDNA exonuclease activity and an endonuclease activity. Although CtIP has been reported to possess an intrinsic nuclease activity, its enzymatic involvement in resection is controversial. However, CtIP is recruited to DSBs via its interaction with NBS1 and it enhances the endonuclease activity of the MRN complex on the 5′ terminated strands of linear dsDNA [116]. MRN–CtIP-dependent end resection in S and G2 is activated by the cyclin-dependent-kinase-mediated phosphorylation of CtIP [116]. In a second phase, the short tracks are extended by the 5′–3′ dsDNA exonuclease activity of exonuclease (EXO)1, or by the combined action of Bloom syndrome RecQ-like helicase (BLM) and DNA replication helicase/nuclease (DNA)2 (long-range resection) [115]. EXO1, DNA2, and BLM contribute also to repair by SSA since long-range resection is a prerequisite for this process as well [22,70]. The homologous regions might be considerably apart in some cases, and SSA can require resection extending for few hundred thousand base pairs.

In contrast to HR and SSA, repair by A-EJ requires the exposure of much shorter tracks of homology in proximity of the break site. Accordingly, limited resection is necessary to enable A-EJ (15-100 nucleotide 3′ overhangs) [22]. Genetic studies have shown that DNA end processing in this context is also mediated by the MRN–CtIP pathway [25,116], whereas the involvement and extent of dependency of A-EJ on EXO1 and DNA2 are still unclear. Furthermore, A-EJ is active throughout the cell cycle and is therefore dependent on resection taking place outside S/G2 phases as well. Indeed, recent studies have shown that DSB end resection occurs also in G1 phase, albeit to a more limited extent [117,118]. This DNA end processing is dependent on the DSB-induced, Polo-like-kinase-3-mediated phosphorylation of CtIP, which activates the MRN-CtIP resection pathway in G1 [117,119]. Of note, some end-joining reactions involving CtIP-dependent resection in this phase of the cell cycle are repaired by the NHEJ pathway [120].

The 3′ ssDNA tails generated by the DNA end processing described previously are coated with and stabilized by the ssDNA-binding factor replication protein A (RPA), a heterotrimeric complex comprising RPA70, RPA32, and RPA14 subunits.

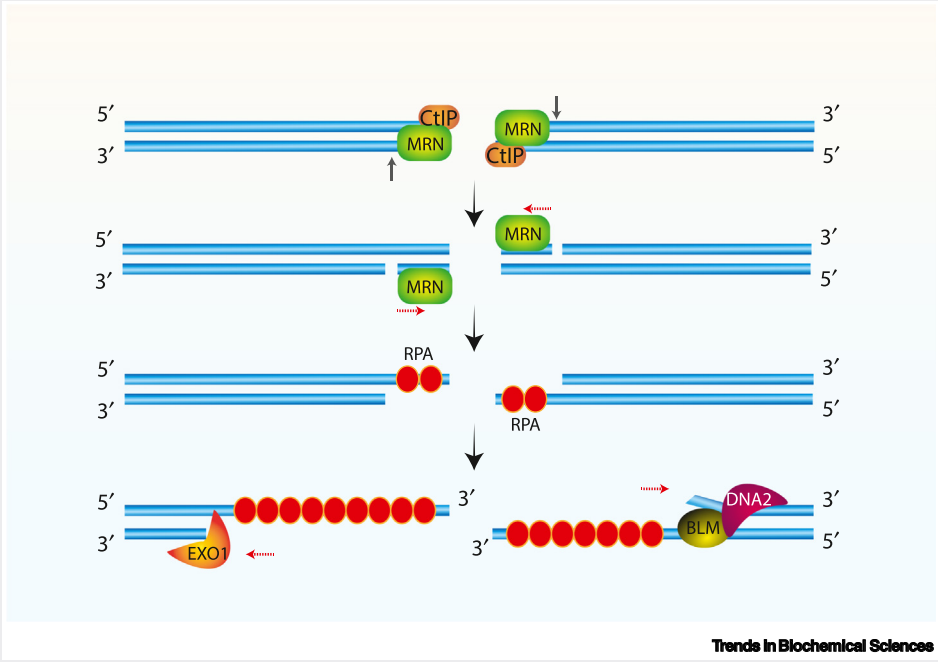

Figure I. 5′–3′ DNA End Resection Occurs via a Bidirectional Two-Step Mechanism. Studies with functionally homologous proteins in yeast suggest that during the initial short-range resection phase, MRE11 first introduces a nick on the 5′ end filament in the vicinity of the double-strand break using its 5′ to 3′ endonuclease activity in a CtIP-dependent manner. Subsequently, it degrades the same strand from the nick towards the break site using its processive 3′–5′ exonuclease activity [115]. MRE11-generated nicks create also an entry site for the enzymes and factors promoting long-range resection, which is mediated by EXO1, or BLM and DNA2 [115]. EXO1 resects the DNA using its 5′–3′ double-stranded DNA exonuclease activity. BLM helicase activity unwinds the DNA duplex, and the 5′ strand is degraded by the endonuclease activity of DNA2. The 3′ single-stranded DNA stretches generated by resection are coated by RPA, and become the substrate for RAD51 loading during HR, RAD52-mediated annealing during SSA, and POLθ-dependent strand pairing in alternative end joining [25,70,114]. Gray arrows denote endonucleolytic activity whereas red arrows indicate the direction of exo-/endonucleolytic processing. Abbreviations: BLM, Bloom syndrome RecQ-like helicase; CtIP, CtBP-interacting protein; DNA2, DNA replication helicase/nuclease 2; EXO1, exonuclease 1; MRN, MRE11–RAD50–NBS1 complex; RPA, replication protein A.

events bearing >1-bp insertions at the junctions [53]. Functional redundancy might explain the complete lack of CSR efficiency and microhomology usage phenotypes observed in the absence of POLθ, which provides gap-filling, end-tethering, and strand-pairing activities during A-EJ in other cellular contexts [24,55]. In reference to the latter activity, a recent study identified 5hmC binding embryonic stem cell-specific protein (HMCES) as a novel DNA strand pairing factor operating exclusively in the A-EJ pathway during CSR [56]. B cells lacking HMCES exhibit a mildly reduced defect in CSR in an isotype-dependent manner, and a junction profile skewed towards direct joins [56]. Furthermore, combined HMCES- and Ku-deficiency nearly abrogates CSR in CH12 cells [56]. HMCES can bind 3′ and 5′ DNA overhangs in a sequence-independent manner [57,58], and it has been proposed to present the ssDNA ends in a configuration that would favor strand annealing during MMEJ, while simultaneously protecting them from excessive resection [56].

The final step in A-EJ-repair of AID-induced breaks is mediated by LIG1 and LIG3. The two ligases have redundant roles in A-EJ during CSR, since LIG4-independent CSR is not affected by inactivation of either ligase or XRCC1 [59–61]. However, LIG1 and LIG3 mediate CSR with

approximately tenfold slower kinetics than LIG4 [62], which is likely caused by the additional requirement of DNA end resection during A-EJ.

In addition to the CSR defect and accumulation of *Igh* locus breaks, NHEJ-deficient B cells display an increased frequency of chromosomal translocations involving the *Igh* locus [28,31]. Junctions from these aberrant repair events present a bias towards the use of microhomologies [31,63]. Altogether, these observations indicate that although NHEJ is the dominant end-joining mechanism for repair of AID-induced DSBs, CSR can occur also via A-EJ, albeit with slower kinetics and reduced efficiency, which ultimately predispose B cells to aberrant DSB end joining reactions leading to chromosomal translocations.

Contribution of HR to Repair of AID-Induced DSBs

Given the predominantly G1-specific nature of CSR and lack of sufficient sequence homology between the different S regions [18,20,27] (Figure 1), HR is considered largely dispensable for CSR. However, B cells deficient for the RAD51 paralog XRCC2, which lack a functional HR pathway, accumulate AID-induced DSBs at both *Igh* and non-*Igh* loci at postreplicative stages of the cell cycle [64,65]. These findings indicate that although AID-induced breaks are repaired predominantly and efficiently in G1 by NHEJ, *Igh* and off-target DSBs that escape repair can persist into S phase, where they are extensively resected and become substrate for HR [64–66].

Since repair by HR occurs by using the homologous sequence on the sister chromatid to copy the genetic information (Figure 2), this process restores the original sequence (conservative homology-driven repair). The conservative nature of HR repair of AID-induced DSBs has important physiological implications. On the one hand, high fidelity repair of AID-inflicted breaks at off-target genes would safeguard the B cell genome from the deleterious consequences of illegitimate repair reactions. On the other hand, HR repair of *Ig* breaks that failed NHEJ-mediated CSR attempts would restore the original pre-DSB sequence, and provide the B cell with another opportunity to undergo productive CSR in the next G1 phase. In light of this consideration, it is worth noting that mature B cells are fully proficient for the activation of the **G1/S DNA damage checkpoint** following IR, but are tolerant to some extent to breaks specifically induced by AID [65–67]. This phenomenon appears to be linked to the capability of B cells to differentially suppress p53 induction depending on the DNA damaging source (AID vs exogenous), and is in agreement with the finding that p53 transcription is actively repressed in germinal center B cells [65,68,69]. The attenuated G1/S checkpoint activation in response to AID-induced lesions ensures that B cells successfully complete CSR by tolerating these physiological DSBs, while concomitantly preserving the ability to fully activate the checkpoint in response to nonprogrammed sources of DNA damage. Furthermore, it would provide B cells with the possibility to employ HR-mediated repair of DSBs persisting from G1, and restore an intact *Igh* allele for the next round of AID targeting and CSR.

A Role for the SSA Pathway during CSR?

Inter-S-region recombination leading to CSR is not the only possible outcome of AID-induced DSB repair. S-region breaks can also be rejoined locally in a process known as intra-S-recombination [15]. This process leads to sequence loss (by rejoining the same DSB after resection) and internal switch deletions (by joining two different DSBs within the same S region). Intra-S junctions isolated from wild-type cells display frequent microhomologies. Furthermore, deficiency in the core NHEJ components Ku, XRCC4, and LIG4 results in increased intra-S-recombination [31]. These local repair reactions have been therefore considered to be mediated preferentially by the A-EJ pathway. Interestingly, a recent study reported that intra-S-repair is dependent on the SSA factor RAD52 [52].

SSA repairs breaks generated between DNA stretches exhibiting >20–25 nucleotides of homology, which are exposed on either strand by resection and annealed before ligation, thus resulting in the deletion of the intervening sequence (nonconservative homology-driven repair) [22,70] (Box 3 and Figure 2). RAD52 has a robust single strand pairing activity, and promotes the annealing of the homologous ssDNA sequences [22,70]. SSA usually requires extensive resection, and the pathway is considered to be most active in S/G2 phases. However, since SSA uses homologous regions present on the same DNA molecule and does not rely on the sister chromatid template for repair, it can in theory operate throughout the cell cycle.

During CSR, RAD52 competes with Ku for binding to the S region DSB ends, and channels repair into intra-S-recombination, thus resulting in internal S region deletions and reduced inter-S-repair events leading to CSR [52]. If we consider also the internally repetitive nature of the S regions (Figure 1), the substantial length of the repeat units, and the fact that AID induces multiple breaks within the S regions, it is conceivable that the SSA pathway might contribute to some intra-S-recombination events. However, combined deletion of Ku and RAD52 nearly abrogates CSR, thus indicating that RAD52 plays a major role also in A-EJ-mediated CSR [52]. The observations that the homology between different S region repeats is either limited or nonexistent (Figure 1), and that microhomologies at the CSR junction from NHEJ-deficient B cells are usually <10 bp [28–30,35,37], favor the conclusion that inter-S-region repair events are indeed mediated by A-EJ rather than SSA, but still rely on RAD52. Therefore, the operational distinction between A-EJ and SSA at the S regions may not be clearly defined, and it is possible that intermediate lengths of homology are repaired by either pathway.

Regulation of End Joining during CSR

The choice of which pathway to engage to repair AID-induced breaks is not stochastically determined. Rather, it is the result of several factors converging into the preferential repair of these breaks by either NHEJ or A-EJ.

Influence of S Region Homology, DSB Structure, and AID on Pathway Choice

One of the most basic factors that has been suggested to bias repair during CSR is the extent of homology between the recombining S regions. The terminal annealing of microhomologies greatly stabilizes end-joining reaction intermediates. As a consequence, it is conceivable that recombination between Sμ and Sα (Sα1 and Sα2 in humans), which share the highest extent of homology [3,71], relies more on A-EJ compared to CSR events involving the less-homologous acceptor regions (Sγs) [43–46,71]. However, a recent report suggests that the higher frequency of Sμ–Sα junctions with longer microhomologies could still be explained by invoking resection-independent NHEJ-mediated repair [72]. Nevertheless, and in agreement with the hypothesis of the extent of S region homology influencing pathway choice, deficiency in components of the A-EJ pathways are often more apparent when assessing CSR to IgA than to IgG isotypes [48,49,56].

A second important factor influencing pathway engagement is the break structure. AID-induced DSBs are created by BER and MMR-mediated processing of the U:G mismatches into **nicks** and gaps [14] (Figure 1). Relatively close nicks and gaps on opposite DNA strands melt into DSBs of different structures (blunt ends, and overhangs of both polarities, 5′ and 3′, and various lengths). The high density and seemingly random location of AID-induced breaks, coupled with the repetitive nature of the S regions, interfere with the unambiguous identification of the original positions of nicks and gaps, thus making it difficult to assess the impact of DSB structure on pathway choice. However, the relationship between type of CSR break and repair outcome was recently analyzed using **CRISPR/Cas9-based gene editing** approaches to generate DNA lesions

mimicking AID-induced breaks [73,74]. Although this approach does not fully recapitulate AID-dependent DSB formation, it clearly showed that in mouse cells staggered (5′ and 3′) DSBs are more extensively processed than blunt ends, and skew Cas9-mediated CSR towards the A-EJ pathway [73]. Consistent with this observation, decreased levels of AID-induced deamination, which would reduce the density of nicks/gaps and lead to increased formation of staggered DSBs, shift repair towards CtIP-dependent A-EJ [75]. Furthermore, in agreement with the findings in mouse cells, a similar Cas9-based study highlighted the preferential use of 5′ overhangs over 3′ DSBs as substrates for Cas9-mediated CSR and translocations in human cells [73,74], and suggested a differential mode of overhang processing based on break polarity [74]. It has been shown recently that high cellular levels of purine nucleotides shift repair towards an increased use and length of insertions at junctions without affecting microhomology usage, and concomitantly reduce CSR efficiency and *Igh* translocation frequency [76]. A similar phenotype has been observed during Cas9-mediated CSR of staggered DSBs with 5′ overhangs, but not blunt ends [76]. Considering that the conversion of AID-generated U:G mispairs generates a considerable portion of staggered DSBs, these findings suggest that insertional repair negatively impacts processing and joining of AID-induced breaks.

Finally, AID has been reported to influence end joining pathway selection at a post-deamination step [77–79]. Specifically, deletion of AID C-terminal domain does not affect DSB formation at the S regions, yet severely impairs CSR. Furthermore, it results in reduced recruitment of NHEJ factors, and skews DSBs towards extensive resection and the use of longer microhomology at S–S junctions [77–79]. Overall these findings indicate that inter-S-region homology, density, and polarity of AID-induced breaks, as well as post-DSB formation properties of AID, impact repair and pathway choice.

Regulation of DSB End Resection

In addition to being a prerequisite for the initiation of homology-dependent repair (Box 3), 5′ resection of DSB ends actively inhibits NHEJ and predisposes cells to homology-dependent repair [17]. Therefore, DSB end processing represents a key determinant of DSB repair pathway choice.

In mammalian cells, the binding of Ku to the DNA ends protects them from limited processing, whereas the inhibition of extensive resection is actively mediated by the 53BP1–RIF1–Shieldin–CST pathway [17,80,81] (Figure 2). Upon DNA damage, ATM induces a cascade of downstream reactions including ubiquitylation of lysine 15 on histone H2A (H2AK15ub) by RNF168 [80,81]. 53BP1 is recruited to the damaged chromatin via the direct bivalent interaction with H2AK15ub and the constitutively methylated lysine 20 of histone H4 (H4K20me2) [80,81]. ATM-dependent phosphorylation of 53BP1 in turn promotes the recruitment of RIF1 to the break site [67,82–85]. 53BP1 and RIF1 shield DSB ends against 5′–3′ nucleolytic digestion over long stretches of chromatin surrounding the DSB, thus effectively repressing long-range resection [80,81].

The downstream effectors in the pathway have recently been identified. The Shieldin complex comprises four subunits, namely REV7, SHLD1, SHLD2, and SHLD3 [86–92]. The recruitment of Shieldin to damaged chromatin is mediated by the association of SHLD3 to chromatin-bound RIF1, whereas REV7 bridges SHLD2, which it directly binds, and the rest of the complex to SHLD3 [80]. SHLD2 possesses ssDNA-binding activity, which is essential for the complex ability to repress resection [86,89–91]. The RPA-like complex CST (CTC1–STN1–TEN1) interacts with Shieldin and counteracts end resection via POLα primase-mediated fill-in of resected DSBs [93,94]. The precise mechanism by which Shieldin and CST act and cooperate to suppress the formation of long ssDNA is still being investigated.

Inhibition of DNA end resection is an important determinant of CSR efficiency. 53BP1 was recognized as a protein essential for CSR in 2004, as its deficiency severely impairs CSR and increases intra-S-recombination [95–97]. Furthermore, *53bp1*$^{-/-}$ B cells exhibit extensive resection of S region breaks and increased use of microhomologies at repair junctions [26,66,98]. Analogously, depletion/abrogation of RIF1, REV7, and other components of the Shieldin complex causes resection of AID-induced DSBs [67,83,88,99,100] and reduces CSR [67,82,83,86–90,99–101]. Furthermore, CH12 cells heterozygous for a null allele of the CST complex subunit CTC1 display reduced CSR efficiency [94]. Altogether, these findings established that the 53BP1–RIF1–Shieldin–CST pathway promotes NHEJ during CSR by antagonizing long-range DNA end resection.

The CSR defect of B cells deficient for 53BP1, RIF1, REV7, and SHLD2 [67,83,88,95,96,99] is more severe than in cells lacking core NHEJ components [28–30]. One possible explanation is that hyper-resection of DNA ends would, at one point, render them less compatible for repair also by A-EJ. Another nonmutually exclusive possibility is that components of the pathway might contribute multiple functions during class switching. In this regard, although much attention has been devoted to the role of 53BP1 in the regulation of DNA end resection, it is now clear that this factor plays additional functions during CSR, which can be dominant over its role in the regulation of DNA end resection (Box 4). Finally, a recent report showed that 53BP1/Shieldin deficiency leads to loss of Ig expression upon CSR induction, which is caused by frequent deletions of coding sequences in the acceptor C region [99]. However, the study reported that cells lacking the NHEJ factors KU70, KU80, XRCC4, LIG4, and XLF exhibited the same Ig loss phenotype [99]. Therefore, although it is likely that hyper-resection in the absence of 53BP1/Shieldin contributes to these deleterious recombination events, loss of Ig expression can be caused also by inefficient repair of CSR DSBs despite the presence of a functional DNA end protection machinery.

Box 4. CSR Breaks in a Context

CSR is a multilayered process dependent on the interplay between transcription, programmed DNA damage, and DNA repair (see Figure 1 in main text). The coordination of these activities is supported by *Igh* locus-specific conformational features that ensure the integration of these reactions into productive end-joining events leading to CSR. In contrast to repair of random DSBs, *in cis* repair of AID-induced breaks at the *Igh* locus exhibits a bias towards deletional–orientation end joining, which favors class switching [121]. This repair feature is intrinsic to the dynamic reorganization of the *Igh* locus architecture during CSR (Figure I). In resting B cells, the 3′ *Igh* enhancer 3′ regulatory region (3′RR) establishes contacts with regions surrounding the 5′ *Igh* enhancer Eμ [122,123]. Upon activation, specific acceptor S regions are recruited into the Eμ–3′RR loop in a cytokine-dependent manner [123], and this interaction promotes high levels of transcription at the recombining S region [124]. A cohesin-mediated chromatin loop extrusion mechanism then aligns the donor Sμ and acceptor S region to enforce the deletional–orientation joining of AID-induced DSBs [124].

The orientation repair bias at the *Igh* locus is strictly dependent on 53BP1 [121]. Residual junctions from 53BP1-deficient B cells display an approximately normalized ratio of deletional versus inversional end joining, thus indicating that ablation of 53BP1 reduces the deletional bias [121]. This activity is not dependent on 53BP1 function in the regulation of DNA end resection [121]. 53BP1 deficiency causes also changes in the profile of chromatin contacts within the *Igh* locus [125,126]. Furthermore, 53BP1 has been shown to enforce a preferential order of targeting for DSB formation at the S regions, with Sμ being targeted before the acceptor S region, and this function is independent from its role in DNA repair [126]. Altogether, these findings indicate that 53BP1 integrates DNA damage-dependent and independent functions that impact CSR at both a structural and resection regulatory level.

Deletional orientation joining appears to correlate to some extent with NHEJ repair proficiency, as it is also dependent on XRCC4, DNA-PKcs, and the helicase activity of the pro-NHEJ factor excision repair cross-complementation group 6 like 2 (ERCC6L2) [127,128]. Furthermore, the orientation bias is mildly affected by deficiency in RIF1, H2AX, and ATM [121]. The identity of additional players as well as how all these factors contribute to this *in cis-Igh* organization feature have not been fully elucidated yet. In regard to 53BP1, it is possible that its ability to oligomerize might contribute to its structural role(s) at the *Igh* locus, since mutations that interfere with 53BP1 higher-order oligomer formation completely abrogate CSR without substantially affecting DSB end resection [129].

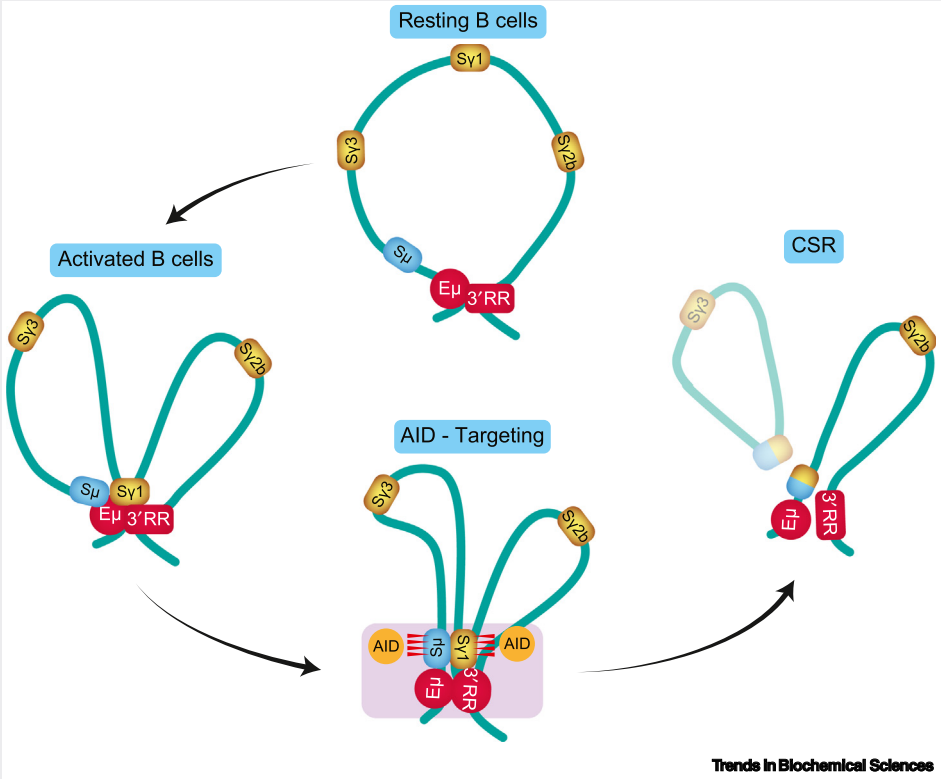

Figure I. *Igh* Locus Architecture Dynamics during CSR. In resting B cells, the 3′RR establishes broad interactions with regions surrounding Eμ, thus forming a characteristic looping configuration (Eμ–3′RR loop). Following activation, the locus architecture dynamically changes as the primed acceptor region is recruited to Eμ–3′RR and recombining S regions are aligned to favor deletional end joining of AID-induced breaks, which leads to productive CSR events. Only few representative S regions are shown in the figure. Abbreviations: 3′RR, 3′ regulatory region; AID, activation-induced cytidine deaminase; CSR, class switch recombination.

Outstanding Questions

Which additional DNA end processing and A-EJ factors operate on S region breaks? How are AID-initiated DSBs of different structure processed prior to ligation?

To which extent does SSA contribute to CSR? What is the relationship between A-EJ and SSA during repair of S regions breaks?

How does AID influence end joining pathway choice? What is the underlying molecular mechanism?

Do Shieldin and CST complexes cooperate to repress the formation of ssDNA during repair of S region DSBs? In which way?

What is the precise contribution of the *Igh* locus architecture to repair of CSR breaks?

How are the different CSR-related functions of 53BP1 coordinated? What is the extent of each function contribution to the overall CSR efficiency?

To which extent is CSR a reliable read-out for DNA end protection proficiency?

Altogether, these observations further highlight the complexity of the regulatory mechanisms that have evolved to ensure efficient antibody diversification by CSR.

Concluding Remarks

The last few years have witnessed remarkable steps forwards in our understanding of the pathways that repair CSR breaks and their regulation. The decision of which pathway to engage to repair S region breaks has crucial implications for both immunity and lymphomagenesis. This choice is influenced by a variety of factors that are either dependent on the DSB formation process, intrinsic to the break structure and recombining S regions, or determined by actively regulated steps in the post-DSB phase. Furthermore, *Igh* locus-specific organizational features greatly impact repair of S region breaks. The in-depth molecular dissection and elucidation of how all of these different regulatory layers converge into productive CSR events will be instrumental to understand how B cells successfully diversify antibody genes while preserving the integrity of their genome in the process (see Outstanding Questions).

CSR has been increasingly used as a direct read-out for NHEJ, and much emphasis has been given in the last years to the correlation between defects in the regulation of DNA end resection and impaired CSR. However, CSR is a complex process that relies on the coordinated interplay

of chromatin reorganization, transcription, programmed DNA damage formation and repair. It is therefore important to keep in mind that DSB repair in this context is embedded in a framework of reactions that have to be tightly coordinated, and caution should be adopted when inferring the function of DNA repair factors from the impact of their deficiency on CSR. Nonetheless, the investigation of the molecular bases of CSR is not only essential for our understanding of humoral responses, but it provides an invaluable framework to assess the physiological and systemic consequences of deficiencies in DSB repair pathways.

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