Immunoglobulin class switch DNA recombination: induction, targeting and beyond

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Abstract

Class switch DNA recombination (CSR) of the immunoglobulin heavy chain (IgH) locus is central to the maturation of the antibody response and critically requires the AID cytidine deaminase. CSR entails changes of the chromatin state and transcriptional activation of the IgH locus upstream and downstream switch (S) regions that are to undergo S-S DNA recombination, induction of AID, and targeting of CSR factors to S regions by 14-3-3 adaptors and as enabled by the transcription machinery and histone modifications. In this Review, we focus on recent advances in CSR induction and targeting. We also outline an integrated model of the assembly of macromolecular complexes that transduce critical epigenetic information to enzymatic effectors of the CSR machinery.

Antibodies (immunoglobulins) are central mediators of immunity[1]. They directly neutralize pathogens and pathogen products as well as recruit molecular and cellular immune effectors to eradicate infections and tumor cells. IgM, IgD, IgG, IgA and IgE antibody classes are identified by different constant regions in their heavy chains (IgH) and have distinct tissue distribution and efficacy against different types of pathogens. IgMs are secreted as pentamers or hexamers, possess a high avidity for antigens with repetitive motifs (such as those occurring on most microbial pathogens) and mediate complement activation. They, however, cannot pass into the extravascular space due to their large size. By contrast, monomeric IgG, monomeric IgE and monomeric or dimeric IgA can distribute systemically to tissues where they mediate a variety of biological effector functions[2]. While naïve B cells express only IgM and IgD, selected immunoglobulin classes and/or subclasses (isotypes) of antibodies are elicited during the course of an immune response, depending on the nature of the eliciting antigen and its entry mode. In humans, IgG1 and IgG3 are effective against viruses, IgG2 against encapsulated bacteria[3], IgG4 and IgE against large extracellular parasites[4], and IgA1 and IgA2 against pathogenic bacteria at the mucosae[5].

The constant regions of different immunoglobulin isotypes are encoded by different C_H exon clusters, which are organized in the order of $C\mu$, $C\delta$, $C\gamma$, $C\varepsilon$ and $C\alpha$ in the IgH locus (FIG. 1). Class switch DNA recombination (CSR) results in the replacement of the expressed C_H exon cluster — for example, $C\mu$ for IgM — with $C\gamma$, $C\alpha$ or $C\varepsilon$, thereby giving rise to IgG, IgA or IgE, respectively, in which the antigen-binding variable region is unaltered[6, 7]. Of note, IgD is generated not through CSR, but through alternative splicing of the primary transcripts that encode IgM. CSR also gives rise to class-switched B cells, such as IgG^{+} B cells, which can respond to antigen faster than naive Igμ^+ or Igδ^+ B cells, probably because the longer cytoplasmic tail of IgG induces stronger B cell receptor (BCR)

signaling[8]. Together with somatic hypermutation (SHM), which inserts mainly point-mutations in the antibody variable region at a high rate to provide a structural substrate for positive selection of higher affinity mutants by antigen[9, 10], CSR is central to the maturation of the antibody response elicited by natural infections and vaccines[11–13]. Defective or aberrant CSR results in diseases ranging from hyper-IgM (HIGM) syndrome, systemic or organ-specific autoimmunity, allergy and asthma associated with atopic IgE, to neoplastic transformation.

In this Review, we focus on the molecular mechanisms underlying the induction of CSR and targeting of the CSR machinery to the upstream and downstream S regions that are to undergo S-S DNA recombination by the RNA polymerase II transcriptional machinery and adaptor proteins that specifically bind repeats characteristic of S region DNA. We also discuss the emerging scaffold functions of both enzymatic and non-enzymatic elements in the stabilization of CSR factors on S regions and recent data on the epigenetic induction and regulation in the specification of the S region targets of CSR.

S region DSB generation and resolution

CSR requires transcription that initiates at an intervening (I_H) promoter and elongates through the I_H exon, switch (S) region and then the C_H exon cluster, known as germline I_H-S-C_H transcription. CSR also requires activation-induced cytidine deaminase (AID)[14], which deaminates deoxycytosines in S region DNA, yielding deoxyuracils[15] (BOX 1, FIG. 2). Processing of such deoxyuracils results in insertion of double-strand DNA breaks (DSBs) in the upstream (donor) and downstream (acceptor) S regions. CSR then proceeds through DSB resolution, leading to deletion of intervening DNA, which gives rise to an extrachromosomal DNA switch circle, and S-S junctions that bring the downstream C_H region DNA closer to the V_HDJ_H region DNA.

To mediate CSR, AID and other factors must be significantly upregulated and then targeted to S regions that will undergo recombination, where they generate DSBs and ultimately promote S-S DNA recombination. The generation of DSBs (which are obligatory CSR intermediates) entails processive AID-mediated deamination of deoxycytosines, particularly those within 5′-AG$\underline{C}$T-3′ and 3′-T$\underline{C}$GA-5′ tandem repeats in the 'core' of the S region, yielding high densities of deoxyuracils in both DNA strands (FIG. 2). Uracils are removed by uracil DNA glycosylase (UNG), a critical element of the base excision repair (BER) pathway, to yield abasic sites. Excision of these abasic sites by apurinic/apyrimidinic endonucleases (APEs) leads to single-strand DNA breaks (SSBs). Proximal SSBs in opposite strands would readily form staggered DSBs[6]. In addition, staggered DSBs can arise from AID- and UNG-dependent processing of blunt S region DSBs generated by endonuclease G[16, 17]. Finally, topoisomerase I can introduce nicks in S region cores, likely by cleaving non-B form DNA[18].

AID can deaminate DNA flanking the S region core, but less efficiently due to the paucity of 5′-AGCT-3′ repeats, thereby leading to generation of mainly SSBs. Such SSBs can be converted to DSBs in a manner dependent on key mismatch repair (MMR) elements such as MSH2 and MSH6. These element may be recruited to deoxyuracil:deoxyguanine mismatches adjacent to the SSB nicks, where they recruit 5′→3′ exonuclease I (EXO I) to yield DSBs[19] (FIG. 2). Thus, MSH2 adds to the main BER pathway (which is dependent on the S region core and UNG) to generate DSBs, as emphasized by the ablation of CSR in B cells lacking both UNG and MSH2[20] or in B cells lacking both the Sμ core and MSH2[21].

DSBs in S regions are generated under physiological conditions, but likely trigger the same set of DNA damage response (DDR) factors that repair DSBs generated by extrinsic DNA-damaging agents, such as ionizing irradiation. DSBs are eventually resolved by the classic

non-homologous end joining (C-NHEJ) or the alternative end joining (A-EJ) pathway (BOX 2). A prominent role of C-NHEJ, which occurs in the cell cycle G1 phase, in CSR is supported by the predominant presence in the G1 phase of AID-dependent S region DSBs and IgH foci that contain the CSR factor NBS1[6, 22]. Of note, homologous recombination occurs in the G2/M phase and it is unclear when A-EJ occurs. A-EJ, which is mediated by CTIP[23] and PARP1[24] (TABLE 1), functions as a back-up pathway when a core factor of C-NHEJ is absent or occurs when DSBs are generated through the MSH2- and EXO I-dependent pathway in CSR[25]. Overall, resolution of S region DSBs in CSR is a highly coordinated process that utilizes the DNA repair machinery and maintains genomic integrity.

Induction and regulation of CSR

Induction of CSR requires both "primary" and "secondary" stimuli. Primary CSR-inducing stimuli, whether T-dependent or T-independent, induce expression of AID and other important CSR-related genes through transcription factors, such as nuclear factor-κB (NF-κB; FIG. 3). Secondary CSR-inducing stimuli, that is, interleukin-4 (IL-4), transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ, in mice but not humans), cannot induce AID, but are required for directing switching to IgG, IgA and/or IgE by selecting the acceptor S region(s) through induction of germline I_H-S-C_H transcription.

During T-dependent antibody responses, CSR occurs mainly in germinal centers and is induced upon engagement of CD40 on B cells by trimeric CD154 expressed by CD4$^+$ follicular T helper (T_{FH}) cells[26, 27]. CD40 induces high levels of CSR to IgG1 and IgE in the presence of IL-4 and moderate levels of CSR to IgG2a and IgA in the presence of IFN-γ and TGF-β, respectively[28]. Deficiency in CD154 or CD40 results in HIGM syndrome in humans and greatly reduced IgG, IgA and IgE levels in mice[6], indicating a critical role of CD40 in class-switched T-dependent antibody responses. Nevertheless, specific IgG and IgA antibodies can emerge at early stages of an infection before specific T helper cells are activated, suggesting that T-independent and CD40-independent CSR has a role in the generation of these antibodies. In addition, T-independent CSR mediates the generation of IgG antibodies specific for many microbial antigens, such as bacterial polysaccharides. Indeed, T cell- or CD40-deficient mice display normal serum IgG3 levels and can generate IgG antibodies that are protective against a variety of infectious agents, albeit not as efficiently as wild-type mice[29].

Deleterious mutations in Toll-like receptors (TLRs), TACI or their downstream signaling molecules result in reduced polysaccharide-specific IgG antibodies and defective immune response to encapsulated bacteria[30], suggesting that T-independent CSR is induced by TLRs and/or TACI. However, engagement of TLRs[31] or TACI[32, 33], with or without IL-4, TGF-β or IFN-γ co-stimulation, induces marginal levels of CSR. The BCR signal, which alone does not induce CSR, synergizes with signals from TLR1–TLR2, TLR4, TLR7 or TLR9 to efficiently induce CSR to IgG3 and, in the presence of IL-4, TGF-β or IFN-γ, to other immunoglobulin isotypes; it also enhances TACI-dependent IgA switching[33]. The only known microbial component that can directly induce efficient CSR is bacterial lipopolysaccharides (LPS). LPS can engage both TLR4 on mouse B cells through its lipid A moiety and can crosslink the BCR of a large proportion of B cells through its repetitive polysaccharidic moiety (O-antigen)[33]. The need for dual TLR and BCR engagement for CSR induction emphasizes the role of naturally linked microbe-associated molecular patterns and repetitive microbial antigens in shaping the adaptive B cell response[34]. Like the BCR signal, the TACI signal synergizes with the signal from TLR7 and TLR9 to induce CSR[32, 33].

Overall, CD40 engagement mediates T-dependent CSR, whereas dual TLR–BCR, TACI–BCR or TLR–TACI engagement mediates T-independent CSR, which plays an important role in T-independent antibody responses but also in early stages of T-dependent antibody responses.

Induction of AID

Whether induced in a T-dependent or T-independent manner, CSR requires AID, which is expressed in a B cell lineage-specific and differentiation stage-specific fashion. AID is virtually undetectable in resting mature B cells but is expressed at high levels in switching B cells. In addition, AID is expressed at a low level and can mediate CSR to IgE in immature B cells[35]. It might also mediate epigenetic programming in primordial germ cells (PGCs)[36] and reprogramming in somatic cells[37], particularly in light of the recent evidence that AID (and APOBEC family members) can promote demethylation of 5-hydroxymethylcytosines (5hmCs, converted from 5-methylcytosines by TET hydroxylases) when coupled with the BER pathway, which removes 5-hydroxymethyluracils (likely generated by AID-mediated deamination of 5hmCs)[38]. A tight regulation of AID expression is necessary to avoid chromosomal translocations and maintain genomic integrity in both B cells and non-B cells[39–41]. This is achieved through fine control of the AID gene (*AICDA* in the human and *Aicda* in the mouse) transcription[19, 42], post-transcription[14] and post-translation (including nuclear/cytoplasmic distribution[43] and stability[44, 45]) regulation and enzymatic function[46].

Transcription of the AID gene depends on NF-κB, which is activated in B cells mainly by primary CSR-inducing stimuli, that is, CD40, TLR and BCR[33, 34], and TLR and TACI[47] (FIG. 3). Of note, CD40, TLRs and TACI, but not BCR, alone can induce AID expression. CD40 engagement and dual TLR–BCR engagement can activate both canonical and non-canonical NF-κB pathways[33, 48]. Owing to its ability to engage TLR4 and crosslink BCR, LPS activates both pathways to induce AID, as highlighted by the recruitment of the (non-canonical) NF-κB p52 subunit to the AID gene promoter[28] and the (canonical) p65 subunit to an upstream enhancer element[49]. Also, the kinetics of AID induction (peaking at 48 – 60 h after stimulation) mirror the kinetics of activation of the non-canonical NF-κB pathway[33], which mediates sustained gene expression to support cell proliferation (required for CSR) and differentiation[50]. By contrast, the canonical NF-κB pathway is rapidly activated to induce immediate, but typically transient gene expression. Thus, activation of the canonical and non-canonical NF-κB pathways initiates and sustains AID gene transcription.

In addition to NF-κB, which is ubiquitously expressed and broadly regulates many genes[51], transcription factors that are expressed in a B cell lineage- and/or differentiation stage-specific fashion[42, 49] regulate AID induction. Prominent among these is HOXC4, a homeodomain transcription factor that is highly expressed in B cells and is induced by the same stimuli that induce AID and CSR. HOXC4 directly binds to the AID gene promoter, which is evolutionarily conserved, through a site (5′-ATTT-3′) embedded within a binding site for POU domain-containing transcription factor OCT1 and/or OCT2[28] (5′-ATTTGAAT-3′). NF-κB and SP1 also bind the same promoter[28] (FIG. 3). HOXC4 is upregulated by estrogen, thereby potentiating AID and CSR[52, 53], suggesting that estrogen is responsible for the AID dysregulation in systemic lupus erythematosus (SLE) patients, as well as in lupus-prone mice. These mice display increased levels of class-switched (and hypermutated) autoantibodies and chromosomal translocations, which are induced in a HOXC4- and AID-dependent fashion[54].

AID induction is influenced by four major evolutionarily conserved regions in the AID gene locus through selected *cis*-regulating elements, many of which are binding sites of transcription factors that are activated by IL-4 and TGF-β (FIG. 3). For example, signal transducer and activator of transcription 6 (STAT6), which is activated by IL-4, probably

binds Region I located upstream of the promoter, and IL-4-activated STAT6 and TGF-β-activated SMAD3 and SMAD4 bind Region IV located 9 Kb upstream of the promoter[49]. In addition, paired box protein 5 (PAX5) and E2A proteins bind Region II within the first intron[49, 55], and the AP1 family transcription factor BATF binds Region III located 17 Kb downstream of the promoter[56, 57]. These transcription factors likely interplay with NF-κB and HOXC4 on the promoter and enhancer elements, probably through long-range DNA interactions, to mediate the AID induction by primary CSR-inducing stimuli and cytokines.

The AID gene transcription is subjected to negative regulation through four repressive *cis*-elements in Region II: two putative binding sites for MYB, which is known to activate or repress transcription depending on gene targets, one putative binding site for the E2F transcription factor family, which contains both activators and repressors, and a 350-basepair CT-rich sequence[49]. Both MYB and inhibitory factors in the E2F family are expressed in naïve B cells and non-B cells and would, therefore, play an important role in silencing AID expression in those cells. In switching B cells, this silencing effect is either reversed or overcome by primary stimuli- and cytokine-activated transcription factors. Finally, AID expression is suppressed by inhibitory E-box protein ID2, which antagonizes PAX5 and E2A[42].

Thus, positive and negative transcription factors restrict AID induction to B cells undergoing CSR and/or SHM.

Germline I_H-S-C_H transcription specifies CSR

Primary stimuli enable CSR but require the cooperation of secondary stimuli to direct switching to predetermined immunoglobulin isotypes. Thus, secondary stimuli (IL-4, TGF-β or IFN-γ) adapt antibody effector functions to different milieus, such as IgA in the mucosae, where TGF-β is produced at high levels[58, 59]. CSR specification to a given immunoglobulin isotype depends upon induction of germline I_H-S-C_H transcription that progresses through the related acceptor S region – the donor S region is constitutively transcribed. During transcription elongation, the chromatin accessibility of intronic S regions is greatly increased[60], thereby facilitating the targeting of the CSR machinery. This reflects the general principle that an open chromatin state is required for DNA recombination, repair and replication. Germline I_H-S-C_H transcription would generate single-strand DNA in the non-transcribed strand in S regions for AID-mediated deamination of deoxycytosines[61, 62]. During transcription, the RNA exosome will allow AID to deaminate deoxycytosines in the transcribed DNA strand, possibly by degrading RNA molecules in RNA:DNA hybrids[63].

Germline I_H-S-C_H transcription is initiated by specific I_H promoters in a manner generally dependent on cytokine-activated transcription factors. IL-4 activates STAT6, which binds the Iγ1 and Iε promoters to initiate germline transcription that proceeds through Sγ1 and Sε for CSR to IgG1 and IgE, respectively. TGF-β activates SMAD3, SMAD4 and/or RUNX proteins to promote transcription proceeding through Sγ2b and Sα for CSR to IgG2b and IgA, respectively. IFN-γ activates T-bet, STAT1 and/or STAT2 for the initiation of germline transcription that proceeds through Sγ2a for CSR to IgG2a[6, 62]. Competition between different I_H promoters for the transcription machinery furthers isotype selection. For example, IKAROS mediates suppression of germline Iγ2a-Sγ2a-Cγ2a and Iγ2b-Sγ2b-Cγ2b transcription, thereby downregulating CSR to IgG2a and IgG2b, and promotes class-switching to IgG3 and IgG1[64].

Primary and secondary stimuli activate distinct sets of transcription factors. The division of roles of these factors, however, is blurred due to their combinatorial interplay on promoters and enhancers in the IgH locus. IL-4, TGF-β or IFN-γ can only induce significant levels of germline I_H-S-C_H transcription when CD40 or TLR and BCR are also engaged. Such

transcription is induced at levels sufficient for CSR only in the presence of a fully functional IgH intronic enhancer (iEμ)[65] and the locus control region located 3′ of Cα (3′-LCR)[66]. This contains several DNase I-hypersensitive enhancer elements that can be activated by NF-κB, HOXC4, OCT1 and/or OCT2[67], suggesting that these transcription factors likely interact with those activated by IL-4, TGF-β or IFN-γ through long-range DNA "synapsis" involving 3′-LCR and selected I_H promoters for full induction of transcription[68]. The combinatorial interplay of primary and secondary stimuli-induced transcription factors is also important in AID induction. IL-4-activated STAT6 and TGF-β-activated SMAD significantly enhance AID induction by NF-κB, HOXC4, OCT1 and/or OCT2 activated by primary CSR-inducing stimuli.

Thus, while primary CSR-inducing stimuli activate transcription factors that regulate transcription of AID and a variety of genes, secondary stimuli (IL-4, TGF-β or IFN-γ) activate transcription factors that bind to different I_H promoter(s) to initiate germline I_H-S-C_H transcription, thereby specifying the acceptor S regions that are to undergo CSR. Full induction of germline I_H-S-C_H transcription requires the interplay of both sets of transcription factors.

Targeting of the CSR machinery

The specific targeting of the CSR machinery to the S regions that will undergo recombination relies on: the recurrence at a high frequency of 5′-AGCT-3′ repeats in S regions, but not in the genome at large, that are specifically bound by 14-3-3 adaptors and other CSR elements; the induced open chromatin state of the S regions targets of recombination (as specified by secondary CSR-inducing stimuli), allowing for access of the CSR machinery; and, finally, the stalling of RNA polymerase II during germline I_H-S-C_H transcription, allowing for high rate deposition on those S regions of the AID hitched by the polymerase.

S regions and 5'-AGCT-3' repeats

S regions are the specific targets of the CSR machinery, and deletion of Sμ or downstream S regions ablates CSR[69]. The CSR machinery does not insert DSBs at predetermined sites within S regions. Rather, it can introduce DSBs in virtually any area of an S region (which ranges from 3 Kb to 10 Kb), but with a strong preference for its core. The core of all S regions is highly enriched in 5′-AGCT-3′ repeats. These account for over 45% of Sμ core DNA but less than 1.4% of DNA in the genome, including C_H regions[70]. The S region-specific and frequent recurrence of 5′-AGCT-3′ repeats is conserved across species that use CSR to diversify their antibodies, from frogs to humans[70] (SUPPLEMENTARY FIG. 1). High serum levels of specific immunoglobulin isotypes in certain species (for example, IgE in horses and IgG2a in rats) likely reflect adaptation to selection applied by pathogens in host living environments or diets; the high 5′-AGCT-3′ frequencies in S regions of these isotypes are, therefore, suggestive of an evolutionary benefit of 5′-AGCT-3′ tandem repeats to CSR.

CSR is impaired in B cells in which the Sμ core was deleted and is almost abolished in B cells that compound the deletion of the Sμ core with abrogation of MSH2[21]. Furthermore, replacement of the mouse Sγ1 DNA with the 5′-AGCT-3′-rich Sμ DNA from *X. laevis* promoted CSR to IgG1[71] and replacement of the full-length mouse Sγ1 region with Sγ1 and Sγ3 cores, which are rich in 5′-AGCT-3′ repeats, was sufficient to mediate CSR[69]. These observations further support the critical importance of 5′-AGCT-3′ repeats in CSR. In addition, 5′-AGCT-3′ is one iteration of the 5′-RGYW-3′ hotspot of SHM and contains the AID deamination motif 5′-WRC-3′ on both DNA strands[72]. 5′-AGCT-3′ repeats provide the substrate for AID to generate deoxyuracils at high-densities on both DNA

strands and eventually DSBs in the S region core. Given the paucity of 5′-AGCT-3′ repeats in areas flanking the S region core, AID-mediated deamination in those areas, likely dependent on the R-loop formation[73], would lead to generation of mainly SSBs, which are converted to DSBs in a MSH2-dependent manner[6].

Thus, the 5′-AGCT-3′ core repeats provide the structural substrates for the intrinsic affinity of the CSR machinery for S regions but not for other DNA sequences in the IgH locus or non-immunoglobulin genes in the genome at large.

14-3-3 proteins as targeting adaptors

5′-AGCT-3′ repeats are not merely preferred substrates of AID, but also the specific targets of 14-3-3 adaptor proteins, which do not bind other 5′-RGYW-3′ iterations or G-rich motifs[70]. 14-3-3 adaptors are recruited to S regions (FIG. 2), and their binding to S regions is blocked by difopein, resulting in diminished CSR. Also, B cells deficient in 14-3-3γ or that express a dominant negative 14-3-3σ mutant are defective in CSR[70].

14-3-3 adaptors play an important role in targeting the CSR machinery to S regions by virtue of their ability to bridge proteins with DNA or other proteins[74]. They directly interact with AID and PKA catalytic subunit (PKA-Cα) and mediate binding of AID and PKA-Cα to the S region(s)[70], where PKA phosphorylates AID[75] (FIG. 2). Binding of 14-3-3 to AID depends on the C-terminal region of AID[70]. This region is dispensable for the deamination activity of AID, but is critical for CSR and has been hypothesized to be the binding site for CSR-specific "cofactors" of AID[76, 77]. The failure of an AID C-terminal truncation mutant (AID$_{1-190}$), which is found in some patients with HIGM syndrome, to bind 14-3-3 adaptors explains the inability of such an AID mutant to be concentrated on S regions[78]. Indeed, an enforced "binding" of AID$_{1-190}$ to 14-3-3γ through physical linking of two peptides could fully rescue CSR in *Aicda*$^{-/-}$ B cells (Mai, T., E.J. Pone, G. Li, J. Moehlman, Z. Xu and P. Casali. 2012. Submitted for publication). By contrast, linking other peptides, such as those from protein kinase inhibitor α, HIV-1 Rev, HTLV-1 Rex, MAP kinase, or Ran binding protein 1, to AID$_{1-190}$ failed to do so[79], suggesting that 14-3-3 adaptors are the AID (C-terminus-binding) cofactors necessary for CSR.

Thus, the inherent S region targeting by the CSR machinery depends on 14-3-3 adaptors, as highly specific for 5′-AGCT-3′ repeats. 14-3-3 adaptors play an important role in the recruitment/stabilization of AID and PKA to/on S region DNA.

Transcription and CSR targeting

The high density of 5′-AGCT-3′ repeats in all S regions and the inherent high avidity of 14-3-3 adaptors for those repeats ensure that 14-3-3 can bind any S regions. These adaptors (as well as other CSR factors), however, only target the donor and acceptor S regions that will undergo recombination[70]. This is likely due to an open chromatin state of these S regions that allows for access of CSR factors, as reflected by the (constitutive or induced) germline I$_H$-S-C$_H$ transcription of the donor and acceptor S regions.

A direct role for germline I$_H$-S-C$_H$ transcription in AID targeting was suggested by the selective association of AID with RNA polymerase II and the transcribed S region DNA[80, 81]. Accordingly, CSR is impaired in B cells deficient in AID-binding proteins that also function in RNA metabolism[63, 82–85], such as SPT5 and SPT6, which regulate transcription, PTBP2, which regulates RNA splicing, and the RNA exosome, which functions in RNA processing (FIG. 4). AID interaction with RNA polymerase II, which is stalled/paused in S regions[86, 87], perhaps due to complex secondary DNA conformations (such as cruciform-like structures) made possible by palindromic 5′-AGCT-3′ repeats, is mediated by SPT5, which, like RNA polymerase II, is enriched in S regions[83]. SPT5,

however, is also enriched on the promoter of a significant proportion of actively transcribed non-immunoglobulin genes[83], consistent with findings showing genome-wide AID binding to transcribed genes[88]. Findings of SPT5- and RNA polymerase II stalling-mediated AID recruitment would lend support to the originally proposed model that the (AID-centered) SHM machinery is linked to the transcriptional machinery[89] and explain recent data that AID can generate mutations in many transcribed genes[90]. AID binding to S regions and selected non-immunoglobulin loci, coupled with the ability of the RNA exosome to allow AID to deaminate deoxycytosines in both transcribed and non-transcribed DNA strands[63], would also explain AID-mediated generation of DSBs in S regions and, at a much lower frequency, throughout the genome[91, 92]. DSBs that are not resolved as intra-S or inter S-S region recombinations will provide the substrate for interchromosomal translocations[93, 94]. These can be deleterious, particularly when the translocation involves oncogenes, such as *MYC*, as occurs at high frequencies in B cell lymphoma, further emphasizing that AID expression, activity and targeting need to be tightly controlled.

Thus, highly regulated mechanisms must be in place to target AID to intronic S regions, which display the highest density in the genome of bound AID molecules. AID may associate with RNA polymerase II through SPT5, then "rides" on the transcription machinery to "scan" the transcriptome until stalled on S region DNA. There, AID is stabilized by binding to 14-3-3 adaptors (FIG. 4). This is reminiscent of the targeting of p53, which uses a non-core domain to bind "non-specifically" to and quickly slide along DNA until "hitting" a target sequence that is then bound by the p53 core domain with high affinity[95]. 14-3-3-mediated AID stabilization in S regions may, in turn, contribute to further stalling/pausing and, therefore, enrichment of RNA polymerase II in S regions.

Thus, targeting of the CSR machinery to the S regions that are to undergo recombination is made possible by the open chromatin state of those S regions. This is enabled by germline I_H-S-C_H transcription and ensures that 14-3-3 adaptors access 5′-AGCT-3′ repeats only in the S regions that are to undergo recombination - all other S regions being inaccessible. Stalling of RNA polymerase II in S regions during transcription allows for deposition of AID, which is further concentrated by 14-3-3.

Epigenetics and protein scaffolding

The recruitment, stabilization and enzymatic function of AID, UNG and other CSR enzymatic elements on S regions require additional interactions, which involve the non-catalytic domains of these enzymes, non-enzymatic adaptors, such as 14-3-3 proteins, and specific histone modifications that are induced in the S region targets of CSR.

Scaffold functions of 14-3-3 and RPA

In CSR, targeting of AID, insertion of DSBs and resolution of DSBs depend on the formation of S region DNA-containing macromolecular complexes. These would be nucleated by the high-avidity binding of 14-3-3 homo- and hetero-dimers or tetramers to 5′-AGCT-3′ tandem repeats in the S region core. Further, as scaffold proteins[96], 14-3-3 stabilize the binding of AID and PKA to S region DNA and enhance AID deamination activity[70]. Within the same macromolecular complex, PKA-mediated phosphorylation of AID at Ser38 *in situ* recruits RPA[88], which enhances AID deamination of deoxycytosines[97]. RPA also likely function as a scaffold protein to stabilize CSR factors in the post-deamination stage, such as UNG[98], MMR factors[99] and the MRE11/RAD50/NBS1 complex[100] in the generation of DSBs, as well as γ-H2AX[101], 53BP1[102] and DNA-PKcs[103] in the resolution of DSBs. In supporting an important role of RPA scaffold functions in CSR, RPA interacts with the UNG N-terminal region, which is critical for CSR[104].

Scaffold functions of AID and REV1

In addition to their deamination activity, AID molecules function as scaffold elements to recruit other CSR factors, such as UNG, MSH2 and MSH6, to S regions and/or stabilize them there. AID molecules are, in turn, stabilized by these factors[78]. The reciprocal and cooperative stabilization of AID with UNG, MSH2 and MSH6 occurs without a direct interaction of AID with these factors and depends on the AID C-terminal region[78]. This region also mediates AID binding to 14-3-3, suggesting that 14-3-3 proteins, which can directly interact with UNG (Lam, T. and P. Casali, unpublished observation), bridge AID and UNG to form an AID–14-3-3–UNG complex. This could enhance the processing of AID-inserted deoxyuracils by UNG for the eventual generation of DSBs.

AID and UNG may play a role in stabilizing factors involved in C-NHEJ over those involved in A-EJ in the DSB resolution stage, as suggested by the increased microhomologies, which are hallmarks of A-EJ, in $S\mu$-$S\alpha$ junctions in patients expressing the C-terminal truncation mutant AID_{1-190} or deficient in UNG[105]. Thus, the AID C-terminal region mediates CSR by targeting AID to S regions through 14-3-3 adaptors, leading to insertion of DSBs, and by mediating scaffold functions that allow for the stabilization of DNA repair factors, which mediate DSB resolution[78, 105, 106]. These contrasting activities of the AID C-terminal region would provide an explanation for the finding that the levels of S region DSBs in B cells expressing AID_{1-190} were comparable to those in B cells expressing wildtype AID[106].

Like AID, UNG mediates CSR in a fashion dependent on both catalytic and non-catalytic amino acid residues[104]. In addition to 14-3-3 and RPA, the translesion DNA synthesis polymerase REV1 directly interacts with UNG through the non-catalytic UNG $_{231}WXXF_{234}$ motif and recruits UNG to S regions to mediate CSR (Zan, H., C.A. White, L. Thomas, J. Zhang, G. Li, E.S. Yu, Z. Xu, T. Mai and P. Casali. 2012. Submitted for publication). Thus, UNG would be stabilized on S regions by RPA through N-terminal domain, by 14-3-3 through C-terminal domain (in a fashion independent of UNG $_{231}WXXF_{234}$ motif) and by REV1 through $_{231}WXXF_{234}$. Interestingly, the catalytic activity of REV1 (incorporation of deoxycytosines opposite deoxyuracil and abasic sites) is important in SHM[107] but dispensable in CSR[108], suggesting that REV1 functions only as a scaffold protein in this process.

Overall, both enzymatic elements and non-enzymatic adaptor proteins exert scaffold functions in CSR through multivalent DNA–protein and protein–protein interactions, thereby cooperatively stabilizing each other as well as other factors on S region DNA (FIG. 4).

Histone modifications in S regions

Adaptor molecules and enzymes are selectively recruited to and stabilized specifically on the S regions that are to undergo recombination because only these regions are in an open chromatin state. This is a reflection of germline I_H-S-C_H transcription as well as loss of repressive histone modifications, such as H3K27me3, and acquisition of permissive histone modifications, such as H3K4me3, in acceptor S regions ($S\mu$ is "constitutively" marked by such modifications in any IgM-expressing B cell)[60, 83, 86, 88, 109–112]. These modifications play an important role in CSR in a fashion independent of germline I_H-S-C_H transcription, as suggested by defective CSR to IgA in mouse CH12 B cells upon siRNA-mediated downregulation of histone methyltransferases and subsequent impairment in H3K4me3 in spite of normal germline $I\alpha$-S-$C\alpha$ transcription[112].

Permissive histone modifications are induced by primary CSR-inducing stimuli in acceptor S regions specified by secondary stimuli, such as IL-4 or TGF-β, which alone cannot induce

such modifications. The concurrent induction of histone modifications (histone code "writing") and germline I_H-S-C_H transcription suggests a coordinated regulation of both processes by transcription factors that specifically bind to the I_H promoters (FIG. 4). Such transcription factors may recruit PAX-interacting protein 1 (PTIP), which plays a critical role in initiation of germline I_H-S-C_H transcription and in generation of multiple histone modifications (H3K4me3, H3K36me3 and H3K27ac, but not H3K4me1) in S regions[60]. In addition, contrasting with the paucity of permissive or activating histone modifications (particularly H3K4me3) in the body – as compared to promoters – of transcribed genes in the genome at large[113], such histone modifications are enriched in S regions at levels higher than those on I_H promoters and exons as well as C_H exons. This difference could stem from the recruitment/stabilization of histone modifying enzymes by stalling of the RNA polymerase II complex and are, therefore, enriched in S regions during germline I_H-S-C_H transcription.

According to the "histone code" hypothesis, combinatorial patterns of histone modifications not only encrypt information on the specification of distinct chromatin states but also increase the complexity of chromatin-interacting effectors[114], thereby determining specific biological information outputs. High levels of many histone modifications in S regions that will undergo recombination suggest that these modifications play a role in recruiting and stabilizing CSR elements to S regions. Among histone modifications in S regions, H3K9me3 (a nominally repressive histone modification) is bound by HP1, as complexed with KAP1 and AID on Sμ, but not any acceptor S regions; B cells deficient in KAP1 display reduced AID binding to Sμ and defective CSR[115]. 14-3-3 adaptors bind the combinatorial H3K9acS10ph histone modification, facilitate histone crosstalk[116, 117] and likely are readers of specific histone modifications in S regions (FIG. 4). 14-3-3 stabilization on S regions through binding to 5′-AGCT-3′ repeats and H3K9acS10ph would parallel the RAG1–RAG2 complex recruitment to V(D)J recombination centers through binding to recombination signal sequences and H3K4me3[118].

Thus, specific histone codes are written in the S regions that are to undergo recombination, resulting from hitching of histone modifying enzymes on RNA polymerase II to reach S regions and enriched there during germline I_H-S-C_H transcription initiated by specific transcription factors, as activated by the combination of primary and secondary CSR-inducing stimuli. Such histone codes are read by CSR factors, such as 14-3-3 adaptors, thereby further stabilizing 14-3-3 on those S regions. Finally, 14-3-3 as well as other scaffold proteins, such as RPA and REV1, stabilize AID and/or UNG on S region DNA and enhance their enzymatic activity, thereby contributing to the "transduction" of epigenetic information to critical CSR factors.

Conclusions

Primary CSR-inducing stimuli provide the main thrust for CSR by inducing AID and other critical CSR factors as well as histone modifications. Secondary stimuli, i.e., cytokines, specify the downstream (acceptor) S region(s) that will undergo germline I_H-S-C_H transcription, histone modifications and, eventually, recombination. This division of roles, however, is blurred, as primary and secondary stimuli synergize – through combinatorial interplay of transcription factors – to induce AID, germline I_H-S-C_H transcription and histone modifications. Better characterization of the recruitment and interplay of these transcription factors would lead to understanding of the dynamics of the IgH locus chromatin opening and accessibility. Induction of open chromatin state and germline I_H-S-C_H transcription in the donor and acceptor S regions allows for 14-3-3 targeting to those S regions, as underpinned by the inherent high avidity of these adaptors for 5′-AGCT-3′ repeats, which are characteristic of all S regions. 14-3-3 adaptors, through direct interactions

with AID and other CSR factors, would nucleate the assembly of macromolecular DNA and protein complexes, within which scaffold enzymatic and non-enzymatic elements further stabilize CSR factors on S regions. The dynamic composition of such macromolecular complexes and nature of histone code readers within the complexes remain to be fully elucidated. The regulation of CSR induction by TLR- and BCR-dependent stimuli and by different cell types needs to be explored, as does the contribution of CSR to the modulation of B cell differentiation. This will likely further our understanding of dysregulation of B cell differentiation and "off-targeting" of AID, which can lead to chromosomal translocations and neoplastic transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary terms

B cell receptor (BCR) signaling	Signals triggered by antigen binding to membrane immunoglobulin of the BCR complex and phosphorylation of CD79a and CD79b of BCR at their ITAM motifs, leading to activation of tyrosine kinases, second messengers and pathways such as NF-κB and MAPK. BCR signaling is important for

	diverse B cell processes including development, survival, activation, proliferation and differentiation.
Hyper-IgM syndrome (HIGM)	Syndromes characterized by elevated titers of IgM and decreased or absent titers of IgG, IgA and IgE, resulting from impairment or ablation of CSR due to B cell intrinsic or extrinsic causes.
Activation-induced cytidine deaminase (AID)	A member of AID/APOBEC cytidine/cytosine deaminase family that catalyzes deoxycytosines deamination to deoxyuracils, thereby initiating immunoglobulin CSR, SHM and gene conversion.
Uracil DNA glycosylase (UNG)	An enzyme that removes uracil bases from DNA by cleaving N-glycosylic bonds and initiates the basic excision repair pathway that removes deoxyuracils from the genome.
Base excision repair (BER)	A DNA repair pathway that replaces damaged bases through excision of damaged bases by DNA glycosylases, nicking of abasic sites by apurinic/apyrimidinic endonucleases (APEs), filling-in of single-stranded DNA gap by DNA polymerases (such as polymerase β) and ligation by DNA ligases.
Endonuclease G (EndoG)	A highly expressed eukaryotic nuclease that cleaves single- and double-strand DNA mainly at deoxyguanines/ deoxycytosines for non-caspase-associated DNA digestion in apoptosis and generation of DSBs in S region DNA in CSR.
Mismatch repair (MMR)	A DNA repair system that recognizes and repairs mismatched DNA base pairs resulting from DNA damage or erroneous insertions, deletions and misincorporations of bases during DNA replication or recombination.
Classic non-homologous end joining (C-NHEJ)	A DNA repair process that joins together non-homologous DNA ends using the four core components KU70, KU86, XRCC4 and DNA ligase IV, and additional components such as DNA-PKcs and Artemis.
Alternative end joining (A-EJ)	A DSB repair process occurring in the absence of certain classical NHEJ components and likely dependent on the MRN complex, CtIP and/or Parp1 to generate a microhomology between DSB ends for end joining.
Homologous recombination	A DNA recombination mechanism whereby extensive homology between two different DNA regions allows for annealing of complementary strands, each from two different regions, followed by enzymatic resolution of this hybrid intermediate, leading to recombination between the two different DNA regions.
Primary CSR-inducing stimuli	Stimuli that induce expression of AID, 14-3-3 adaptors and other important CSR-factors through transcription factors, such as NF-κB, thereby enabling CSR. These stimuli are triggered by ligands engaging B cell surface and endosomal receptors: CD40 (T-dependent stimulus), dual TLR-BCR, TACI-BCR or TLR-TACI engagement (T-independent stimuli). Primary stimuli enable secondary CSR-inducing stimuli to induce germline I_H-

S-C$_H$ transcription and histone modifications in selected switch regions.

Secondary CSR-inducing stimuli — Cytokines IL-4, TGF-β and IFN-γ (in mice but not humans) that cannot induce AID, but are required for directing switching to IgG, IgA and/or IgE by selecting the acceptor S region(s) through induction of germline I$_H$-S-C$_H$ transcription and enrichment of specific histone modifications. These stimuli also enhance AID induction, as enabled by primary CSR-inducing stimuli.

T-dependent antibody response — An antibody response to protein antigens that requires recognition of the antigen by T helper cells and cooperation between antigen-specific B and T cells and leads to the generation of high-affinity antibodies and long-term memory.

Germinal centers — Organized microanatomical sites in secondary lymphoid organs where B cells, upon activation by specific T helper cells, proliferate, undergo SHM and CSR, and differentiate to memory B cells and plasma cells.

Toll-like receptors (TLRs) — A family of conserved PRRs that recognize microbe-associated molecular patterns (MAMPs, invariant molecular structures recurring in microbes, but largely absent in metazoans) through leucine-rich repeats (LRRs) in the extracellular or intra-endosomal domain, and activate signaling pathways, leading to activation and differentiation of innate immune cells and B lymphocytes.

TACI — A TNF receptor family member specific for B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that activates signaling pathways (such as the NF-κB pathway) through TRAF adaptors.

T-independent antibody response — An antibody response to certain polymeric microbial constituents, including those that can directly activate B cell proliferation (type I) and repetitive polysaccharides (type II), in the absence of T cell help.

Systemic lupus erythematosus (SLE) — A chronic systemic autoimmune disease that is characterized by multi-organ pathology including rashes, arthritis and kidney diseases. It is mediated by antibodies that are specific for double-stranded DNA and other nuclear antigens.

Chromosomal translocations — Aberrant chromosome structures that result from the breaking and joining of a chromosome (or part of a chromosome) to another chromosome through improper DSB ligation and that frequently occur in cancers.

RNA exosome — An eukaryotic multi-protein complex comprised of a ring-like nine-subunit core, consisting of six different RNase subunits and three distinct RNA-binding subunits, and three associated subunits that also possess RNase activities. It degrades erroneous RNA molecules, thereby enforcing RNA quality control, and non-erroneous RNA molecules, thereby maintaining their normal half-life and, as recently shown,

	exposing the DNA strands of transcribed S regions for AID to deaminate.
14-3-3 adaptors	A family of seven evolutionarily conserved and highly homologous adaptor proteins that form homo-/heterodimers and/or tetramers and bind a multitude of protein and DNA ligands through either the amphipathic groove or the outer surface. 14-3-3 proteins regulate diverse cell homeostasis events, such as signal transduction, survival, cell cycle progression and DNA replication, and cell differentiation processes, such as CSR.
5′-RGYW-3′ hotspot	A DNA motif that is mutated with the highest possibility, as a result of intrinsic preference of the SHM machinery (R: a purine; Y: a pyrimidine; W: A or T).
R-loop	A nucleic acid structure in which an RNA strand hybridizes with its complementary strand of a double-strand DNA molecule and displaces the other DNA strand that is typically rich in deoxyguanines, thereby forming a loop of up to hundreds of nucleotides.
Histone modifications	Post-translational chemical modifications, including methylation, acetylation and phosphorylation, of histones that are inserted by histone modifying enzymes and can actively mediate specific biological processes by interacting with selected effector proteins, including scaffold proteins that recruit/stabilize downstream factors. Histone modifications also determine chromatin accessibilities for DNA transactions.
Scaffold protein	A multi-domain protein or multi-subunit protein oligomer that, by switching the conformation of its ligands, coordinates the spatio-temporal organization of macromolecular complex, leading to the proximity of enzymes with their substrates and, eventually, specific biological information output.
REV1	A translesion DNA synthesis polymerase that bypasses lesions through its dCMP transferase activity for DNA repair. REV1 also possesses scaffold functions to recruit or stabilize other DNA repair factors.
Histone code hypothesis	A hypothesis postulating that distinct histone modifications that act alone, sequentially or combinatorially, form a "histone code" that is read by effector proteins to mediate biological processes.

Box 1 | Activation-induced cytidine deaminase (AID)

AID is a 198-amino acid protein that initiates CSR and SHM by deaminating deoxycytosines in single-strand DNA and supercoiled double-strand DNA, both of which can arise during transcription. It shares a conserved catalytic domain with other members of the AID/APOBEC family of cytosine/cytidine deaminases. The catalytic domain contains His56, Cys87 and Cys90, which bind Zn^{2+}, and Glu58; the carboxylic acid group of Glu58 serves as a general acid-base catalyst. The APOBEC-like domain of AID binds DNA surrounding deoxycytosines and influences the substrate specificity. The C-terminal domain is essential for AID to mediate CSR and the N-terminal domain is essential for AID to mediate SHM.

Naturally occurring mutations in AID responsible for the autosomal recessive HIGM type 2 (HIGM2) syndrome (the number of cases reported for each mutation is depicted in the box figure) and experimentally generated AID mutations cause defective CSR and/or SHM. AID deamination activity and CSR are virtually abolished by mutations at Arg112 in the APOBEC-like domain and Arg24 in the DNA-binding N-terminal region, which are two positively charged residues frequently mutated in HIGM2 patients. Arg112 is just outside the hotspot recognition loop, which contacts the DNA substrate and determines the substrate specificity of the AID deoxycytosine deamination activity.

The C- and N-terminal domains also function as the nuclear export signal (NES) and nuclear localization signal (NLS), respectively. Active nuclear export of AID results in its dominant cytoplasmic distribution. This together with the instability of nuclear AID limits the levels of AID that can cause genomic damage. AID is phosphorylated at Ser38 and Thr27 to create a binding site for RPA and mediate CSR. Phosphorylation at Thr140 is important for SHM, whereas the role of Ser3 or Tyr184 phosphorylation is unclear. Binding regions in AID to 14-3-3, MDM2 and DNA-PKcs are also depicted.
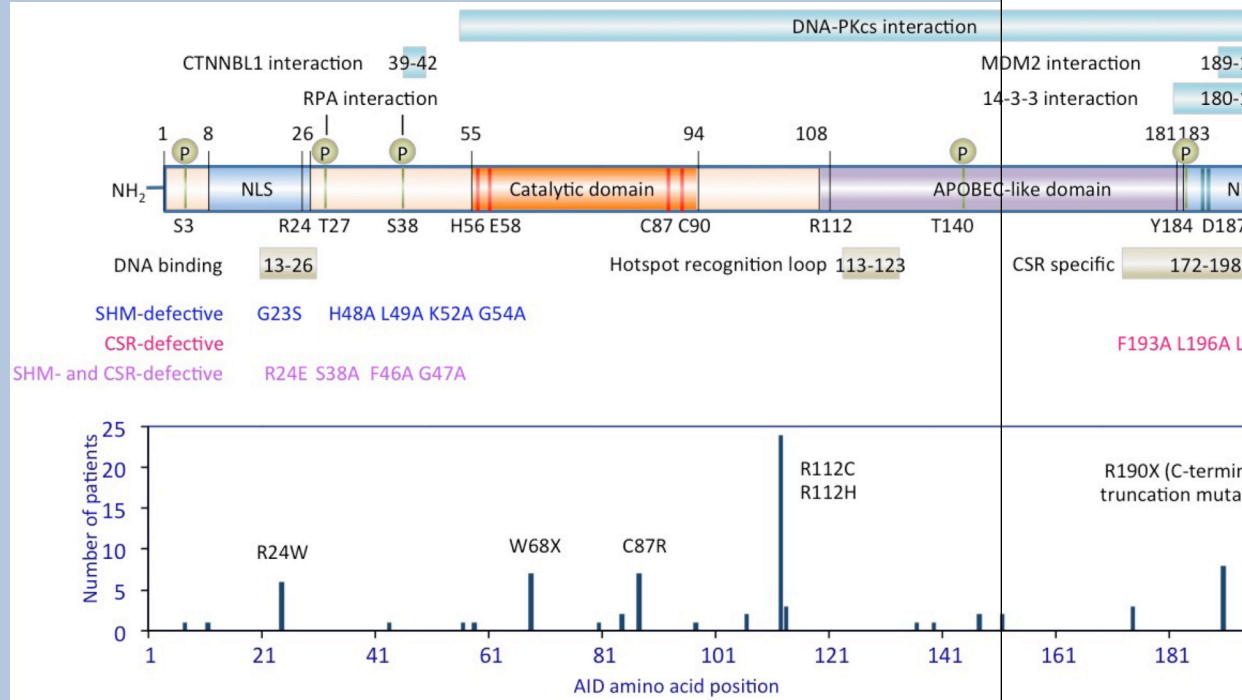
Box 2 | Double-strand DNA break (DSB) resolution in CSR

DNA damage sensors, adaptors and effectors in the DNA damage response (DDR) play important roles in the DSB resolution stage in CSR. The MRE11–RAD50–NBS1 complex (MRN) is an early sensor of AID-mediated DSBs and localizes to damage sites, where it recruits and activates ATM. This phosphorylates NBS1 and downstream DDR proteins, thereby strengthening the DDR response. MRN also activates the γH2AX–MDC1–53BP1 pathway; γH2AX and 53BP1 mediate synapsis of upstream and downstream DSBs. MRN can process DSB ends for both C-NHEJ and A-EJ pathways in a manner independent of the MRE11 nuclease activity (this MRE11 nuclease activity is instead required for DSB repair mediated by homologous recombination). In C-NHEJ, DSBs are bound by KU70 and KU86 that form complexes with DNA-PKcs and play scaffold functions to recruit other essential factors, such as the XRCC4–DNA ligase IV complex, to complete end joining process for the formation of S-S junctions. In A-EJ, DSB ends are processed by MRN and CTIP, thereby generating microhomologies between upstream and downstream DSB ends. Ligation of DSBs by DNA ligase(s), which remain(s) to be identified, yields S-S junctions that show higher microhomologies that those formed in C-NHEJ. The choice of C-NHEJ, which is also essential to maintain genome integrity, over A-EJ, which is frequently associated with chromosomal translocations, depends on the expression of 53BP1, which protects DSB ends from resection for A-EJ, and on KU70 and KU86, which inhibit CTIP. Aberrant repair of DSBs can lead to chromosomal translocations and genomic instability.
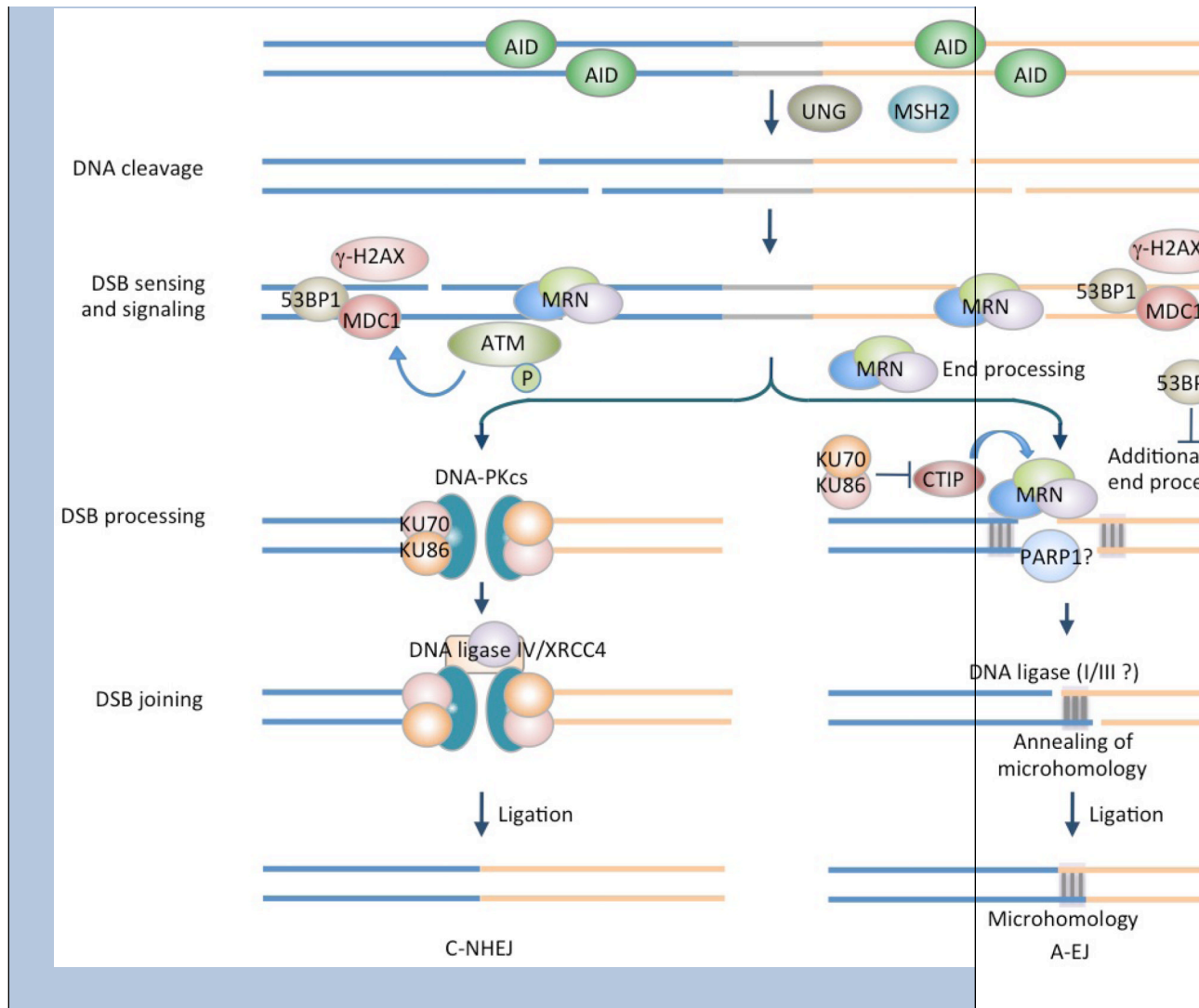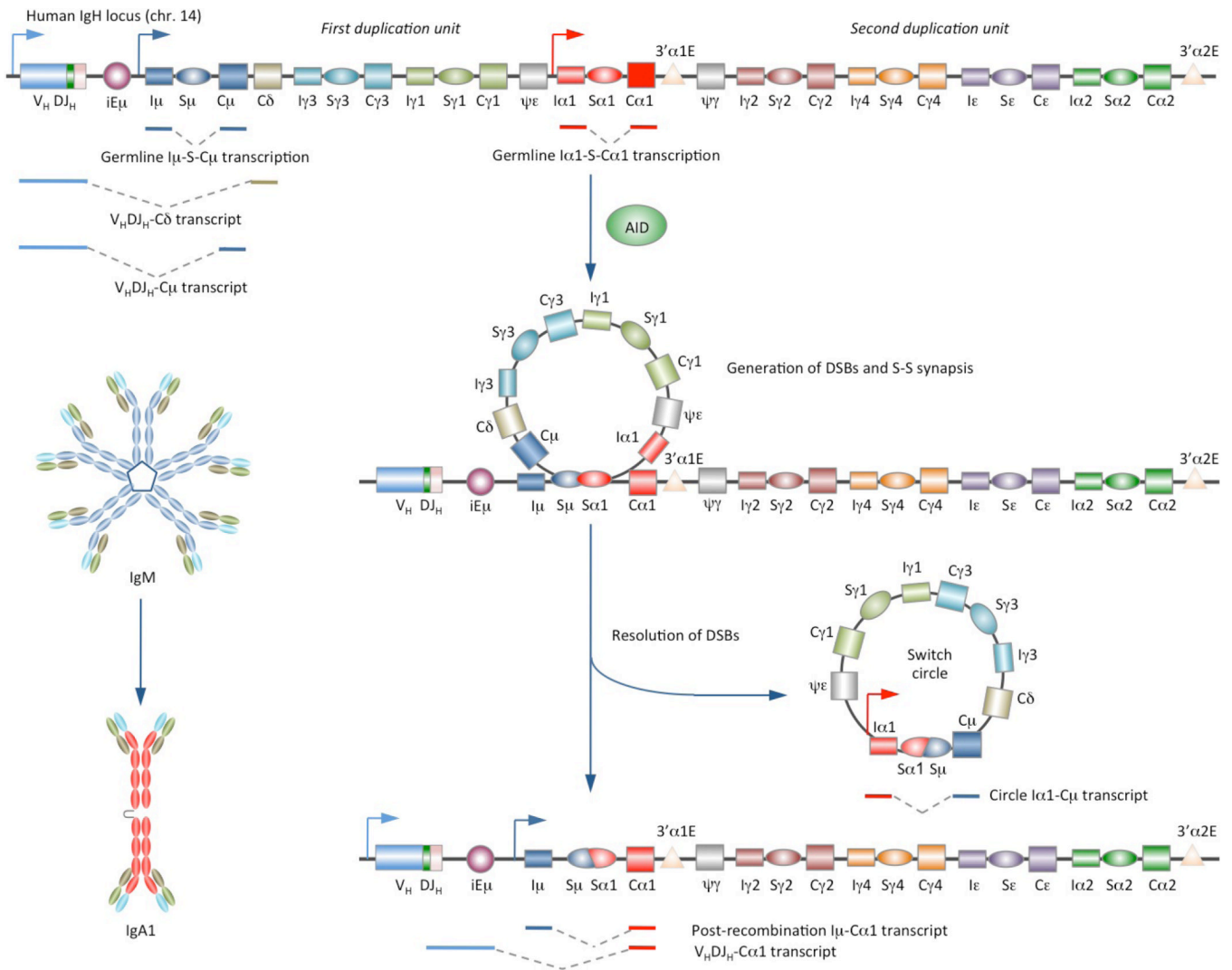
Figure 1. CSR entails DNA deletion

CSR exchanges the gene encoding the heavy chain constant region (C_H) with one of a set of downstream C_H genes (depicted is CSR from $S\mu$ to $S\alpha1$ in the human IgH locus). This deletion-recombination reaction requires AID and involves the generation of double-strand DNA breaks in S regions (lying upstream of the constant-region gene) followed by DSB repair. This leads to juxtaposition of rearranged VDJ DNA with a downstream C_H exon cluster and deletion of the intervening sequence between S regions as an extrachromosomal circle.
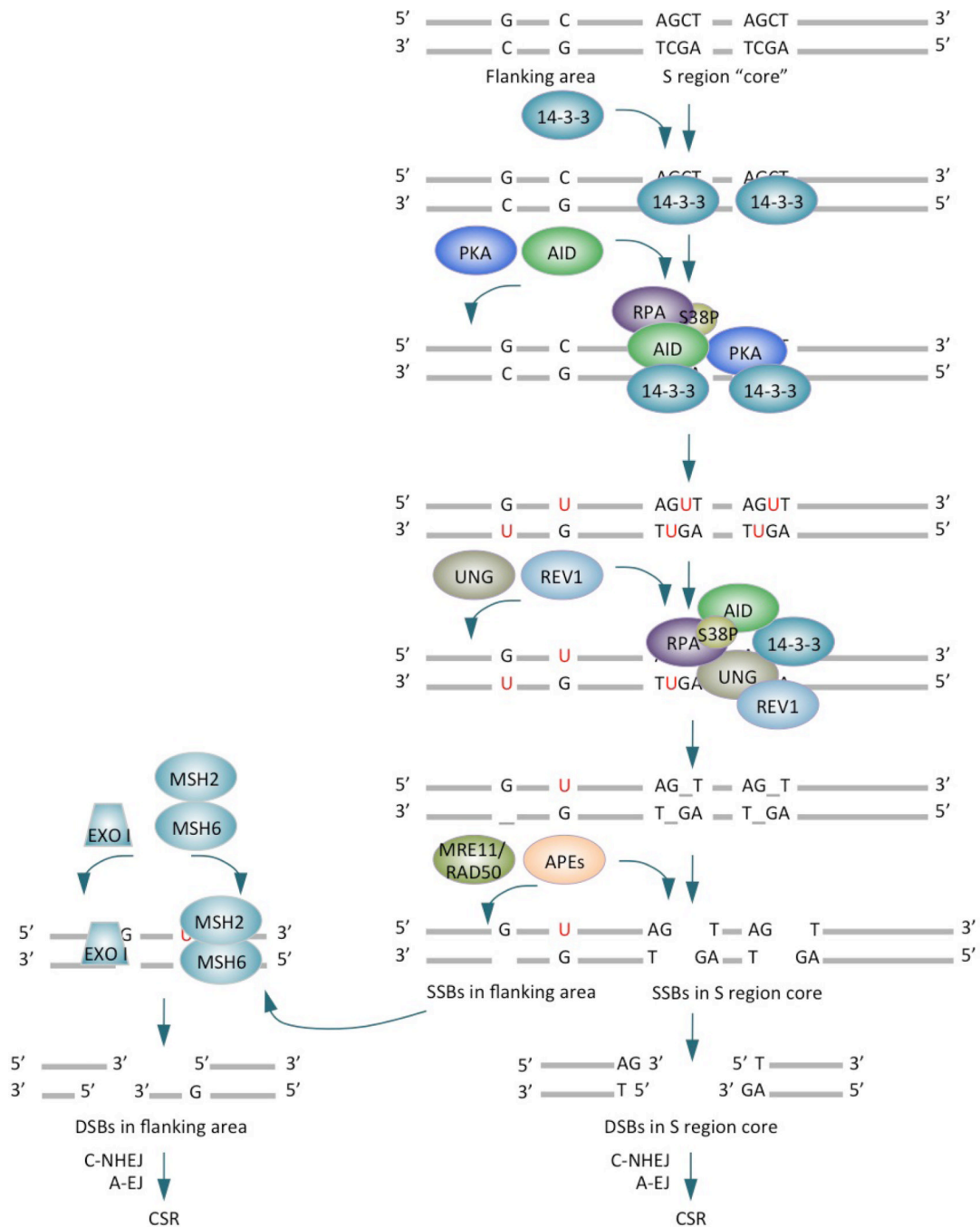
Figure 2. Enzymes and scaffold elements in CSR

CSR is initiated by AID, which is targeted to S regions by 14-3-3 adaptors that specifically bind 5′-AGCT-3′ repeats in the S region core and recruit AID and PKA to S region DNA. AID is phosphorylated at Ser38 of the N-terminal region by PKA, generating a binding site for RPA. AID also binds 14-3-3 proteins through its C-terminal region (inset). RPA enhances AID deamination of deoxycytosines in transcribed S region DNA. The resulting deoxyuracils are removed by UNG, which would be recruited and stabilized on S regions in a fashion dependent on the scaffold functions of 14-3-3, RPA and REV1 (Zan, H., C.A. White, L. Thomas, J. Zhang, G. Li, E.S. Yu, Z. Xu, T. Mai and P. Casali. 2012. Submitted for publication). RPA and REV1 bind to different regions of UNG. UNG is also stabilized

by AID, which indirectly interacts with UNG (likely through 14-3-3, Lam, T. and P. Casali, unpublished observation), within a putative macromolecular complex. Excision of UNG-generated abasic sites by APEs and/or the MRE11–RAD50 lyase results in single stranded breaks, which either directly form double stranded breaks (as occurring in the S region core) or are converted to DSBs in an MSH2- and EXO I-dependent fashion (as occurring in the flanking area). DSB resolution by C-NHEJ or A-EJ leads to formation of S-S junctions and CSR.
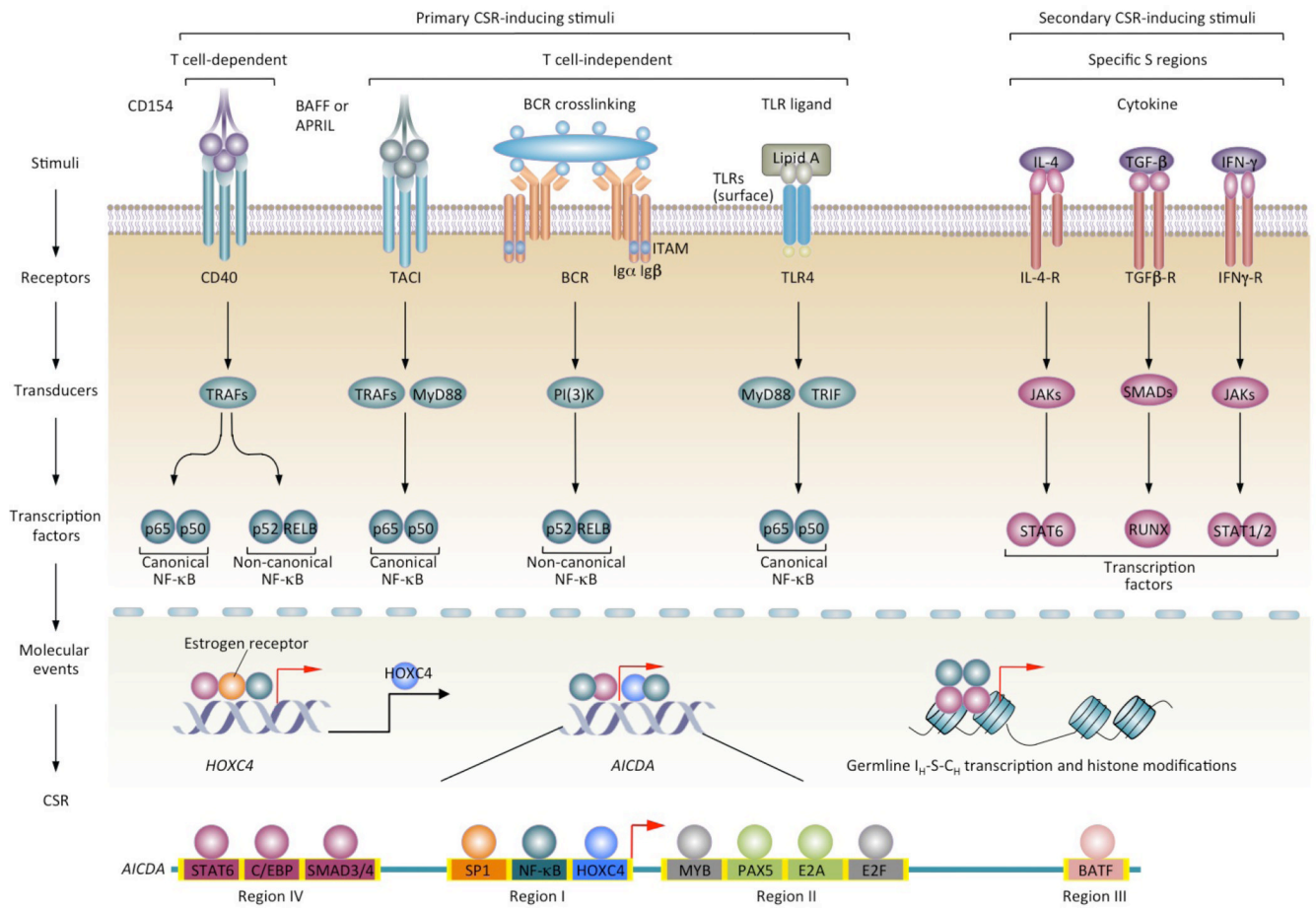
Figure 3. CSR-inducing stimuli and interplay of transcription factors

CSR entails induction of AID, germline I_H-S-C_H transcription and active histone modifications in S regions by primary CSR-inducing stimuli together with secondary stimuli (cytokines). Both T-dependent (CD40) and T-independent (dual TLR–BCR, TACI–BCR or TLR–TACI engagement) CSR-inducing stimuli — through distinct signal transducers — activate NF-κB for induction of HOXC4 (whose expression is enhanced by estrogen) and HOXC4-dependent induction of AID. The ability of primary CSR-inducing stimuli to induce AID and CSR correlates with their activation of both the canonical and non-canonical NF-κB pathways. Cytokines IL-4, TGF-β and IFN-γ do not activate NF-κB, but activate transcription factors specific for I_H promoters. These in turn bind to specific I_H promoters to initiate germline I_H-S-C_H transcription, thereby specifying the S regions that are to undergo recombination. A combinatorial interplay of primary and secondary CSR-inducing stimuli-activated transcription factors (teal and plum circles, respectively) results in optimal induction of AID and germline I_H-S-C_H transcription. Transcription factor-binding sites in Region I through IV of the AID gene locus that have been shown to regulate AID expression are depicted. Binding sites for MYB and E2F (grey circles) negatively regulate AID expression.
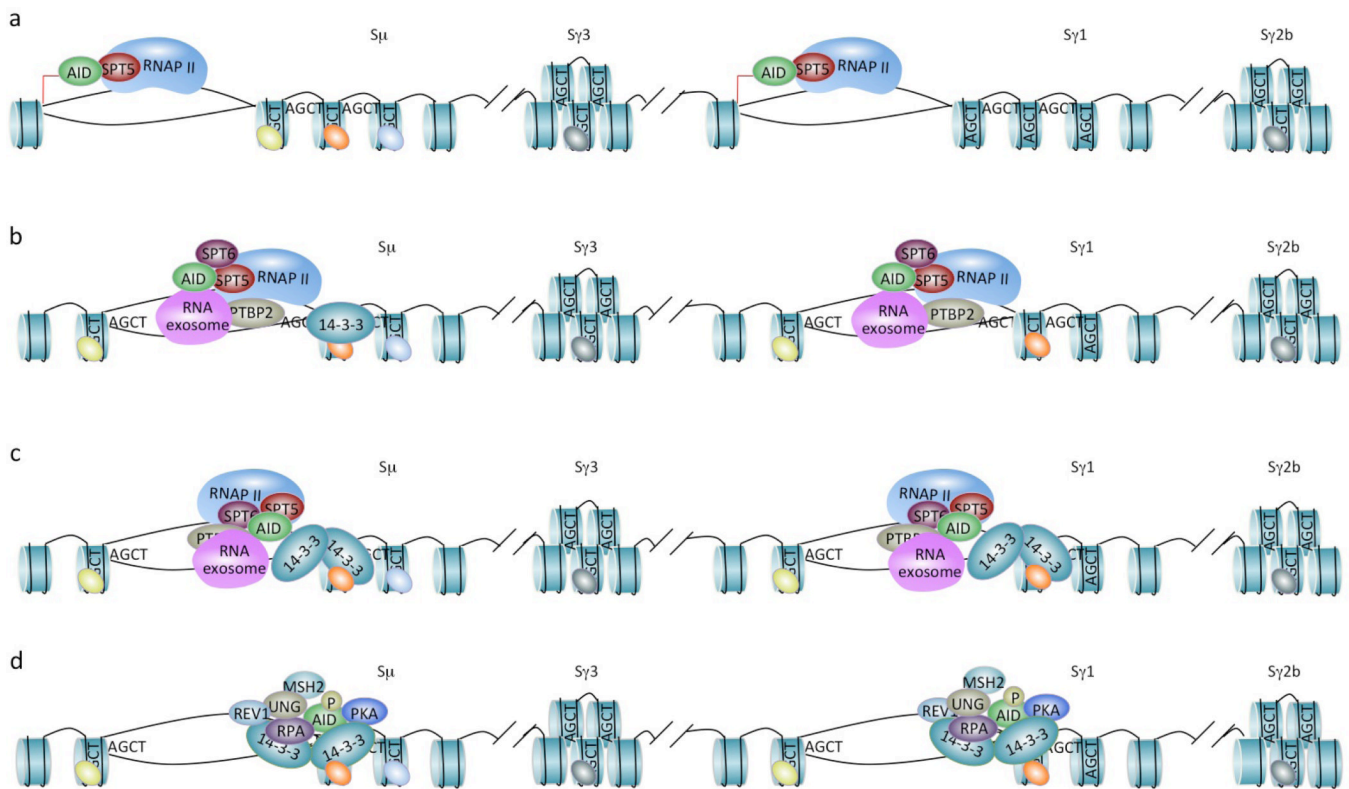
Figure 4. Targeting of the CSR machinery
CSR targeting entails the epigenetic regulation of the S regions that are to undergo recombination (depicted is CSR from Sμ to Sγ1 in the mouse IgH locus), high-avidity binding of 14-3-3 adaptors to 5′-AGCT-3′ repeats and scaffold functions of CSR factors. (**a**) RNA polymerase II and histone modifying enzymes, such as histone methyltransferase and histone acetyltransferase complexes, are recruited to the Iγ1 promoter to initiate germline Iγ1-S-Cγ1 transcription (red arrow) and catalyze histone modifications, respectively (the Iμ promoter undergoes constitutive transcription initiation and histone modifications). Through SPT5, RNA polymerase II associates with AID at the Iμ and Iγ1 promoter, albeit at low levels. (**b**) During transcription elongation, RNA polymerase II together with SPT5 and, perhaps, SPT6, PTBP2 and the RNA exosome (all within a putative macromolecular complex), stalls in Sμ and Sγ1, and likely plays a role in recruiting/ stabilizing histone modifying enzymes, such as histone methyltransferase and histone acetyltransferase complexes that catalyze H3K4me3 (green ovals) and H3K9acS10ph (orange ovals) modifications, respectively. This and loss of repressive histone modifications, such as H3K9me3 (light blue ovals) and H3K27me3 (grey ovals), reflect the open chromatin state of Sμ and Sγ1 that allows the access of AID and other CSR factors. AID, as "hitched" on the transcription machinery, is enriched in S regions due to RNA polymerase II stalling. (**c**) AID is further concentrated to Sμ and Sγ1 by 14-3-3 adaptors, which access the same Sμ and Sγ1 regions due to their open chromatin state and are recruited/stabilized there through interactions with 5′-AGCT-3′ repeats and likely H3K9acS10ph. The RNA exosome also interacts with AID and allows AID to deaminate both transcribed and non-transcribed S region DNA. (**d**) 14-3-3, RPA and enzymatic elements AID and REV1 (Zan, H., C.A. White, L. Thomas, J. Zhang, G. Li, E.S. Yu, Z. Xu, T. Mai and P. Casali. 2012. Submitted for publication) all possess scaffold functions that stabilize each other and other CSR factors, including PKA, UNG and MSH2, on S region DNA within a macromolecular complex.

Table 1

Factors involved in CSR

Factor	(Putative) function(s)	Refs.
	B cell specific factors	
AID	Initiates CSR by deaminating deoxycytosines; involved in post-cleavage repair	6, 7
	Chromatin modification-/transcription-related factors	
PTIP	Promotes chromatin changes that control accessibility of the IgH locus to CSR	60
SSRP1 (SPT16)	Regulates histone modification H3K4me3 and DNA cleavage in CSR	112
	AID-binding factors	
14-3-3 adaptors	Target S regions and recruit/stabilize AID and other CSR factors	70
CTNNBL1	Unclear, might promote AID localization or association with RNA spliceosomes	82
eEF1A	Interacts with AID in the cytoplasm	43
HSP90	Stabilizes cytoplasmic AID	119
KAP1	Interacts with AID and tethers AID to Sμ	115
PKA	Phosphorylates AID at Ser38 on S region DNA	75
PTBP2	Binds RNA and promotes AID binding to S region	85
REG-γ	Interacts with AID and promotes AID degradation in the nucleus	45
RNA exosome	Targets AID to both strands of transcribed duplex DNA	63
RNA polymerase II	Facilitates AID targeting to transcribed DNA	80
RPA	Binds to Ser38-phosphorylated AID and likely facilitates conversion of deoxyuracils to DSBs and promotes repair of DSBs	75
SPT5	Facilitates the association of AID with RNA polymerase II	83
SPT6	Regulates differentially AID functions in CSR and SHM	84
	Base excision repair factors	
APE1/APE2	Nicks abasic site to generate DSBs in S regions	6
UNG	Removes AID-generated deoxyuracils and is involved in the DNA repair stage	20, 104
XRCC1	Likely participates in A-EJ	120–122
	Mismatch repair factors	
EXO I	Generates DSBs by excising DNA containing deoxyuracil: deoxyguanine mismatches as a $5' \rightarrow 3'$ DNA exonuclease.	123, 124
MLH3	Contributes to the creation and/or stabilization of microhomologies between DNA ends in upstream and downstream S regions	125
MSH2, MSH6	Recognizes deoxyuracil: deoxyguanine mismatches and initiates the process that leads to generation of DSBs; stabilizes AID on S region DNA	20
PMS2 and MLH1	Recruit EXO I to promote DNA excision	6
	Endonucleases/nickase	
Endonuclease G	Generates DSBs in S regions in an AID-independent fashion	17
Topoisomerase 1	Introduces nicks in S region, resulting in DSBs	18
	DNA damage sensors, adaptors and effectors	
53BP1	Stabilizes S region synapsis and protects DSB ends from resection	126
APLF	Promotes XRCC4/DNA ligase IV complex retention in chromatin in C-NHEJ	127
ARTEMIS	Mediates resolution of DSBs by C-NHEJ	128–130

Factor	(Putative) function(s)	Refs.
ATM	Mediates DSB recognition and/or repair and S region synapsis	131, 132
ATR	Is involved in end-joining pathways that require long microhomologies	133
NHEJ1	Mediates resolution of DSBs by C-NHEJ	134 135
CTIP	Promotes microhomology-directed A-EJ	23
DNA-PKcs	Plays a role in synapsis and DNA end joining	128
H2AX	Regulates higher order chromatin remodeling that facilitates S region synapsis	136
KU70, KU86	Bind DSB ends and recruit downstream factors; are essential for C-NHEJ	137, 138
MRE11, RAD50, NBS1	Stabilize DSBs and process DNA termini to facilitate repair by both C-NHEJ and A-EJ; also functions as lyase to nick abasic sites	139
PARP1	Facilitates microhomology-directed A-EJ	24
REV1	Recruits/stabilizes UNG to S region DNA	108, 140
RNF8, RNF168	Propagates DSB signals and facilitates the recruitment of various DDR proteins	141–143
XRCC4, DNA ligase IV	Catalyzes the DNA ligation step in C-NHEJ	144, 145

53BP1, p53-binding protein 1; A-EJ, alternative end joining; AID, activation-induced cytidine deaminase; APE, apurinic/apyrimidinic endonuclease; APLF, aprataxin and PNKP like factor; ATM, Ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; CTIP, CTBP-interacting protein; CSR, class switch DNA recombination; CTNNBL1, catenin, beta like 1; DDR, DNA damage response; DNA-PKcs, DNA protein kinase catalytic subunit; DSB: double-strand DNA breaks; eEF1α, eukaryotic translation elongation factor 1α; EXO I, exonuclease I; H2AX, H2A histone family, member X; HSP90, heat shock protein 90; KAP1, KRAB-associated protein 1; KU70, KU autoantigen, 70 kDa; KU86, KU autoantigen, 86 kDa; MLH3, MutL homolog 3; MRE11, meiotic recombination 11; MSH2, MutS homolog 2; MSH6, MutS homolog 6; NBS1, Nijmegen breakage syndrome 1; NHEJ1: nonhomologous end-joining factor 1, also termed XRCC4-like factor (XLF) and CERNUNNOS; PARP1, poly (ADP-ribose) polymerase 1; PKA, protein kinase A; PMS2, postmeiotic segregation increased 2; PTBP2, polypyrimidine tract binding protein 2; PTIP, PAX interaction with transcription-activation domain protein-1; RNF8, ring finger protein 8; RNF168, ring finger protein 168; RPA, replication protein A; SHM, somatic hypermutation; S region, switch region; SSRP1, structure specific recognition protein 1; UNG, uracil DNA glycosylase; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; XRCC4, X-ray repair complementing defective repair in Chinese hamster cells 4.