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Ig heavy chain class switch recombination: mechanism and regulation

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Abstract

Ig heavy chain class switching occurs rapidly after activation of mature naïve B cells, resulting in a switch from expressing IgM and IgD to expression of IgG, IgE, or IgA; this switch improves the ability of antibodies to remove the pathogen that induces the humoral immune response. Class switching occurs by a deletional recombination between two different switch (S) regions, each of which is associated with a heavy chain constant (C_H) region gene. Class switch recombination (CSR) is instigated by activation-induced cytidine deaminase (AID), which converts cytosines in S regions to uracils. The uracils are subsequently removed by two DNA repair pathways, resulting in mutations, single-strand DNA breaks, and the double-strand breaks required for CSR. We discuss several aspects of CSR, including how CSR is induced, CSR in B-cell progenitors, the roles for transcription and chromosomal looping in CSR, and the roles of certain DNA repair enzymes in CSR.

Introduction

After immunization or infection, activated naïve B cells can switch from expressing IgM and IgD on their surface to expressing IgG, IgE or IgA. This isotype/class switch changes the effector function of the antibody, and improves its ability to eliminate the pathogen that induced the response. Isotype switching involves a replacement of the μ and δ heavy chain constant (C_H) regions of the expressed Ig with γ, ε or α C_H regions, and occurs by a DNA recombination event termed class switch recombination (CSR). Fig 1 presents a diagram (not to scale) of the C_H genes and CSR in mice; human C_H genes are similarly arranged although not identical.

CSR is a deletional DNA recombination occurring between switch (S) regions, which are located upstream of all the C_H genes except Cδ, and are one to 10 kb in length (1). Recombination occurs between DNA double-strand breaks (DSBs) introduced into the donor Sμ region and a downstream/acceptor S region located from ~65 to 160 kb downstream, although occasionally downstream S regions can subsequently recombine with a S region farther downstream. S regions are G-rich and also have a high density of WGCW (A/T-G-C-A/T) motifs, the preferred target for activation-induced cytidine deaminase

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(AID), the enzyme that initiates CSR by deaminating cytosines (dC) within S region DNA, converting dC to dU(2, 3). Subsequently, enzymes of the base excision repair (BER) and mismatch repair (MMR) pathways convert the dU's to DNA double-strand breaks (DSBs), which are required for CSR(4, 5) (Fig 2). The DSBs are subsequently recombined by an end-joining type of DNA recombination, predominantly by non-homologous end-joining (NHEJ). The use of NHEJ rather than homologous recombination is consistent with the facts that S region DSBs are induced and recombined during G1 phase (6–9), and that different S regions do not share long stretches of identity (1), which are required for homologous recombination.

CSR occurs very rapidly after infection or immunization, prior to formation of germinal centers, which generally form 7–10 days after exposure to antigen. For example, using mice expressing a transgenic B cell receptor (BCR), both IgM^+ and $IgG2a^+$ cells were detected in B cell follicles from days 2–4 after immunization, but only $IgG2a^+$ cells were detected in germinal centers, indicating that CSR occurred prior to germinal center formation (10). Also, CSR was detected in non-transgenic mice 4 days after infection with *Salmonella* (11). However, CSR is also detected in germinal center B cells from human tonsils (12), and IgA CSR occurs in Peyer's patch germinal centers (13–15), in which B cells are constantly stimulated by the gut microbiota. CSR also occurs during T-independent responses, which do not induce germinal centers (16). Thus, CSR starts prior to somatic hypermutation (SHM) of variable region [V(D)J] genes, an AID-dependent process which occurs mainly in germinal centers, and which, after selection, can result in antibodies with increased affinity for antigen. The data suggest that CSR may continue as long as B cells are undergoing activation.

Induction of CSR

Many studies of CSR have been performed using cultures of mouse splenic B cells, as these cells can be induced in culture to undergo robust CSR within ~3 days by treatment with the B cell mitogen LPS, acting through the innate receptor TLR4, or by signaling through CD40, the most important receptor for T cell help. LPS alone induces AID expression, but a cytokine, such as IL-4 must also be added to induce AID when antibody to CD40 (αCD40) is used. These ligands also induce cell proliferation, another requirement for CSR(17, 18). Surprisingly, splenic B cells do not require a signal provided by the BCR to switch in culture, as neither LPS nor αCD40 trigger signaling via the BCR. This might be explained by the large amounts of LPS (usually 10–50 μg/ml) used in these cultures, which would not normally be provided to B cells in vivo. Also, in these cultures much more continuous CD40 signaling is provided than normally would be available in vivo. In vivo, CD40 signaling would be delivered to antigen-specific B cells during contacts with antigen-specific T_H cells. CD40 signaling is very important for CSR in vivo, as demonstrated by the lack of B cell proliferation and CSR in response to T-dependent antigens in mice and humans deficient in CD40 signaling (19, 20). Also, TLR signaling is important for in vivo immune responses and CSR in response to viruses (21).

Although antigen is not necessary to obtain CSR in culture, addition of low levels (1–100 ng/ml) of soluble hen egg lysozyme (HEL) antigen to LPS- or αCD40-stimulated mouse

splenic B cells expressing a HEL-specific BCR increases CSR(22). Increasing the amount of antigen to 1 μg/ml inhibited CSR. Also, low levels (ng amounts) of anti-IgD (anti-δ dextran added to LPS-activated cultures of splenic B cells increases CSR(23–25). The increase in CSR is not due to increased cell proliferation (24, 26). By contrast, addition of large concentrations (10 μg/ml) of anti-IgM to LPS cultures or αCD40 cultures reduces CSR, although it does not inhibit cell proliferation (27, 28). The inhibitory signaling pathway has been studied, and appears to be a feed-back response to extensive BCR cross-linking.

BCR signaling appears to be important for CSR in culture under conditions when signals provided by accessory/secondary signals are limiting. Ligands for the innate receptors TLR1/2, TLR7, and TLR9 have been shown to induce little or no CSR in culture. When anti-δ dextran is added to these cultures, CSR to several isotypes is increased synergistically (24). Likewise, when HEL is added to HEL-specific B cells activated with TLR7 or TLR9, CSR to IgG1 is also greatly increased (22). Unlike mouse B cells, human peripheral blood or tonsillar B cells switch poorly in culture to either CD40 or TLR signaling (29); perhaps activation through BCR signaling would help.

In conclusion, it is likely that CSR in vivo depends upon activation of B cells via their BCR, in addition to secondary signals from CD40 and TLR signaling. It is unclear whether the BCR signaling is required for inducing CSR or whether BCR signaling is required only for initial activation of the B cell and the T cell signals are responsible for inducing CSR. In addition, it appears likely that TLR signaling is important for both T-independent and T–dependent responses in vivo. It is possible that both primary and multiple secondary signals given together increase the robustness of the CSR response.

Regulation of isotype specificity by transcription of unrearranged C_H genes

Naïve B cells have the potential to switch to any isotype. Isotype specificity is directed by induction of transcription across S regions, as AID-induced deaminations and CSR are restricted to S regions that are undergoing transcription (5, 30–33). Located upstream of each acceptor S region are transcription promoters, which are activated by cytokines that induce CSR to that specific isotype. Transcription from these promoters produces germline transcripts (GLTs), so-called because they are transcribed from unrearranged genes. GLTs are spliced, as diagrammed in Fig 1 for the γ2bGLT. Due to their unusual high G-content, S region transcripts form R-loops (RNA-DNA hybrids) with the bottom strand DNA, rendering the non-transcribed (top strand) single-stranded (ss)(34–36). The substrate for AID is ss DNA; thus, R-loops cause the top strand to become an extensive AID substrate. However, it is known that AID attacks both the top and bottom strand at S regions nearly equivalently (37). Thus, the R-loop must be removed during CSR to expose the bottom strand to AID.

The R-loop is thought to cause RNA polymerase II (Pol II) to stall during transcription of S regions, resulting in accumulation of Pol II in S regions, although some other feature of S regions might be responsible (38, 39). Interestingly, AID has been demonstrated to be in a complex with Spt5, a protein that associates with Pol II when it is stalled on the DNA template, suggesting that Pol II stalling recruits AID to S regions (40). In addition, stalled

Pol II can lead to transcription termination, generating 3′ ends of S region transcripts, which then become substrates for the RNA exosome, a complex which degrades RNA from the 3′ end. The RNA exosome has been shown in biochemical (in vitro) experiments to allow AID to target the bottom strand in transcribed duplex DNA (41), which is otherwise mostly in accessible to AID when associated with newly transcribed RNA. This finding provides an explanation for how AID targets the bottom strand.

These in vitro experiments have been bolstered by in vivo experiments in which CSR was reduced by 50% in cells lacking Nedd4, an E3 ubiquitin ligase. Nedd4 monoubiquitinates paused Pol II, causing an unknown ubiquitin ligase to polyubiquitinate Pol II, leading to degradation of Pol II(42). Pol II degradation leads to transcription termination, creating substrates for the exosome. Interestingly, AID is found associated with polyubiquitinated Pol II, and Nedd4 ubiquitination activity was shown to promote interaction of AID with Spt5 and with the RNA exosome, and to promote binding of the RNA exosome to transcribed S regions. Nedd4 also reduces the level of γ1 GLTs, presumably due to degradation by the exosome. Thus, Nedd4 activity appears to increase the amount of ss bottom strand DNA, and to help recruit AID to S regions. This model has been supported by results indicating that Pol II pausing leads to transcription termination and increased SHM in IgH V genes, dependent on the RNA exosome (43, 44).

GLTs must be spliced to support CSR, although the reason for this is unknown (33, 45–49). As splicing is co-transcriptional, it is possible that splicing factors are involved in recruiting AID to S regions (50), and/or that splicing is required for R-loops to form, perhaps because the RNA must thread back into the DNA to form an R-loop (36). The splicing regulator PTBP2, a protein that binds AID and S region transcripts, is important for efficient CSR. Knockdown of this protein reduces the association of AID to S regions (51, 52). PTBP2 is known to regulate alternative splicing and many aspects of RNA metabolism (53), although its specific role(s) in CSR is not defined. Also, CTNNBL1, a component of the splice some, interacts with AID and is important for both CSR and SHM (50). These interesting studies suggest avenues that might lead to an understanding of why splicing of GLTs is important for CSR, although at this time the role of splicing is not understood.

Regulation of CSR by chromosome looping

Sμ and the acceptor S regions must be in contact in order to recombine. Chromosome-conformation-capture (3C) experiments have shown that in mature naïve splenic B cells prior to CSR, the Eμ intron enhancer (Eμ) and the 3′ Cα enhancer/regulatory region (3′Eα/3′RR), located ~220 kb apart in the genome, are positioned sufficiently near each other in the nucleus to be cross-linked by formaldehyde treatment via proteins bound to them (54–57). Gene targeting experiments have shown that segments of the 3′Eα are essential for CSR to all isotypes (58). This Eμ-3′Eα interaction causes Sμ and the downstream S regions to be located within the same chromosomal loop (Fig 3A). In cells activated to switch, but that have not yet switched to IgG1 by treatment with LPS+ IL-4, the Eμ-Sμ-Cμ and Sγ1-Cγ1 loci are found positioned near each other and the 3′Eα segment (Fig 3B). In cells treated with LPS alone, which induces CSR to IgG3, the Sγ3-Cγ3 locus is associated with Eμ-Sμ-Cμ and with 3′Eα, instead of Sγ1-Cγ1 (54, 55). As LPS+IL-4 induces γ1GLTs, these results suggest

that factors that induce transcription from the GLT promoter and/or the GLTs themselves are involved in recruiting the Sγ1-Cγ1 locus to the positions of the Eμ-Sμ-Cμ locus and 3′Eα. Also, it is possible that looping could allow transcriptional activators that bind the enhancers to gain access to GLT promoters, and thus association of a S-C_H acceptor locus with the Eμ-Sμ-Cμ locus and with 3′Eα might contribute to transcription or even be required for GL transcription.

It is clearly important to identify the proteins regulating loop formation between the Eμ and 3′Eα enhancers and C_H genes in B cells before and during CSR. YY-1, a protein that binds both the 3′Eα and Eμ enhancers is a candidate (59, 60). Better evidence is available for PTIP, which has been shown to interact with the B cell specific protein Pax5, and to be important for the interaction between the 3′Eα and the γ1 GLT promoter (61). Mice deficient in PTIP have reduced γ3, γ2b, and γ1GLTs and reduced CSR to these isotypes (62), but it is unknown how much of this effect is due to reduced interaction between the C_H loci and the 3′Eα, and how much is due to the fact that PTIP is important for recruiting the histone methyl transferase MLL, which produces the activating histone modification H3K4me3. It is possible that MLL and/or H3K4me3 is important for the looping. CTCF and cohesin, two proteins involved in contraction/looping of the V_H and Vκ gene loci (63), do not appear to bind within the 180kb region encoding the C_H genes nor to the 3′Eα. Cohesin, however, has been shown to be required for optimum CSR, but the mechanism of its contribution is unknown (64, 65).

CSR in B cell progenitors

Pre-B cells purified from mouse bone marrow express low amounts of AID and have been shown to have ongoing H chain switch recombination (μ>γ2b), despite the fact that they do not express light chains and IgM (66). This was determined by two molecular assays for switch recombination events at the DNA level, detection of RNA transcripts from the excised DNA circles (circle transcripts), and also post-switch transcripts produced by transcription of the recombined genes. In a different study, pro-B cells from Rag1-deficient mice could be induced in culture to switch at the DNA level from μ>γ2b and from μ>ε, although these cells do not express either μ chains or light chains (67). The physiological role of this H chain switching is unknown, although it might contribute to autoimmunity (68).

Interestingly, differing from CSR regulation in mature B cells, μ>γ2b but not μ>γ3 switching occurs in pro-B cells activated with LPS+CD40 ligand (CD40L), and μ>ε but not μ>γ1 switching occurs in cells treated with LPS+CD40L+IL-4(67). GLT expression correlates with CSR, i.e. γ2b and ε GLTs, but not γ3 or γ1 GLTs, are detected in pro-B cells, activated without or with IL-4, respectively. The explanation for this restricted choice of isotypes appears to be that pro-B cells have a chromosomal loop between the Cγ3 and Cγ1 loci that sequesters these genes away from the Eμ and 3′Eα enhancers (67). This loop was not detected in mature splenic B cells. Likewise, ex vivo bone marrow pre-B cells from C57BL/6 mice switch to γ2b but not to γ3; surprisingly, however, BALB/c pre-B cells switch to γ2b and γ3(66). The pre-B cells in both mouse strains also switched to α, consistent with results indicating that mice that cannot express IgM can undergo CSR to IgA

(69). There are two mature mouse B cell lines (CH12F3 and I.29μ) that can be induced to switch in culture, but the switching is restricted to IgA, or rarely, in I.29μ, to IgE or IgG2a. Perhaps these cell lines have a chromosomal loop that sequesters the other C_H loci from the enhancers. Much more research is needed to understand how specific chromosomal loops are regulated and the roles of chromosomal loops in regulating CSR.

Introduction of DSBs in S regions by AID involves the BER and mismatch repair pathways

Although AID is rapidly induced after B cell activation, the enzymes and proteins that convert the AID-induced dUs to DSBs are constitutively expressed, as the lesions these proteins repair occur in all types of cells. Uracil, whether due to AID activity or caused by spontaneous hydrolysis of cytosine, can be excised by the BER enzyme uracil DNA glycosylase (UNG), which leaves an a basic (apyrimidinic/apurinic) (AP) site (Fig 2A). Although cells express 4 different uracil DNA glycosylases (UNG, Smug1, MBD4 and TDG), deficiency of UNG alone results in a 95–99% reduction in CSR in mice and humans (4, 70, 71), and reduces S region DSBs detected during CSR in cultured splenic B cells nearly to levels observed in $aid^{-/-}$ B cells (72). Recently, it was found that in $ung^{-/-}$ mice, Smug1 can partially substitute, i.e., in $smug1^{-/-}ung^{-/-}$ splenic B cells, CSR is reduced another 5-fold (71). Smug1-deficiency by itself has no effect on CSR, probably due to its low abundance (73)and low activity on dU in ss DNA, whereas UNG is abundant and more active on ss DNA than on ds DNA(74). As AID only has activity on ss DNA, it is possible that UNG excises most dU's prior to reformation of the DNA duplex.

A basic (AP) sites are subsequently cut by AP endonuclease, APE1 and/or APE2, creating a single-strand DNA break (SSB) (Fig 2A). If SSBs are sufficiently near each other on both DNA strands, DSBs are produced. APE1 is essential for cell viability, and although $ape1^{-/-}$ mice have not been produced, $ape1^{+/-}$ mice have DNA repair defects. Both APE1 and APE2 contribute to CSR in splenic B cells induced to switch in culture (75). S region DSBs are greatly reduced in $ape1^{+/-}ape2^{-/-}$ splenic B cells, but only marginally reduced in $ape1^{+/-}$ or $ape2^{-/-}$ cells, indicating these enzymes are partially redundant. However, deletion of these enzymes in CH12F3 B lymphoma cells gave somewhat different results. APE1-deficient cells have an 80% reduction in CSR, but APE2-deficiency has no effect on CSR (76). The difference between results in splenic B cells and CH12F3 cells remains a puzzle.

APE2 is a very inefficient endonuclease, ~1000-fold less active than APE1. APE2 has stronger exonuclease activity than APE1 and thus one could envision APE1 producing the SSB and APE2 creating a gap at the SSB, which could increase the probability that a SSB might become a DSB. However, evidence suggesting that APE2 does act as an endonuclease at AID-induced AP sites in splenic B cells was obtained by determining the locations of S region DSBs in APE-deficient mice compared to WT mice. APE2-deficiency reduced their preference for G:C bp so that the proportion of breaks at G:C bp were not significantly different from their proportion in the sequence itself, whereas DSBs in $ape1^{+/-}$ cells were similar to WT(75). It is not understood why APE2 is important for CSR, especially since APE1 is a much more efficient endonuclease.

A major question in the field is why AID-induced lesions are not accurately repaired despite the fact that BER is a highly active and error-free repair pathway. In most cells, DNA Pol β accurately replaces the excised nucleotide, and Ligase III-XRCC1 seals the phosphodiester backbone, and DSBs are not generated (See Fig 2A). In fact, DNA Pol β modestly inhibits S region DSBs and CSR, suggesting that it competes with DSB formation, but is overwhelmed by the numbers of AID-induced SSBs (77). Another study suggested an additional possibility. In this study, SHM of antibody variable region genes was examined in germinal center B cells from Peyer's patches (78). These cells are undergoing constant stimulation by gut microbes and undergo robust SHM. The results suggest that the use of APE2 instead of APE1 in repair of AID-induced lesions in germinal centers converts BER to an error-prone repair pathway (78). Germinal center B cells express very low levels of APE1 protein and mRNA compared to APE2. APE2 was found to be important for mutations at A:T bp, whereas *ape1*$^{+/-}$ B cells have unperturbed SHM (78). Since a SSB is required as an entry point for the error-prone trans lesion polymerase DNA Pol η to introduce mutations at A:T bp, this suggests that APE2 is indeed acting as an endonuclease during SHM, creating SSBs. It is possible that the use of APE2 might inhibit error-free repair of AID-induced lesions. Interestingly, APE2, but not APE1, interacts with PCNA a protein that recruits DNA Pol η to DNA (79).

When the AID-UNG-APE pathway induces SSBs that are too far apart on opposite DNA strands to produce DSBs, MMR can convert the SSBs to a DSB(5) (See Fig 2B). The Msh2-Msh6 heterodimer binds to U:G mismatches in duplex DNA, recruits Mlh1-Pms2 and Exonuclease 1 (Exo1), which initiates resection from a SSB located 5′ to the mismatch (80, 81). Pms2 also has endonuclease activity and can create additional SSBs on the previously nicked strand, providing additional entry sites for Exo1 or other nucleases (82, 83). As diagrammed in Fig 2B, this resection should result in creation of a DSB with a long ss tail, which when filled in by DNA Pol creates a blunt, or nearly blunt, DSB appropriate for NHEJ(5). Several types of data support this model, including the facts that S region DSBs and CSR are reduced by 2–7 fold in MMR-deficient B cells (7, 84, 85), and that in the absence of Sμ tandem repeats, with their numerous AID target hotspots, CSR is absolutely dependent upon Msh2 (86).

Recombination of S region DSBs by NHEJ

S-S recombination occurs by NHEJ, which involves binding of the abundant toroidal heterodimer KU70/80 to each DSB, forming a platform for nucleases Artemis and PALF, and for DNA polymerases, and greatly stimulating the ligation activity of DNA ligase IV-XRCC4-XLF (reviewed in (87–89)). The Mre11-Nbs1-Rad50 (MRN) complex also rapidly binds DSBs, but it is unclear whether KU and MRN compete or cooperate at S region DSBs. Rad50 has a long coiled-coil domain with a hook at the end by which MRN complexes bound at different DSBs can interact and tether the DSBs to each other (90). MRN recruits additional factors, including the kinase ataxia-telangiectasia-mutant (ATM), which activates and coordinates the cellular response to DSBs. KU and MRN have both been shown to bind S region DSBS (6, 91–93) and contribute to CSR (94–98).

There is a great deal of mechanistic flexibility in NHEJ, which is necessary because DSBs can differ greatly, having 3′ or 5′ ss tails of different lengths, and possibly even having hairpins, in addition to being blunt. Generally, the junctions formed between two S regions have 0 or 1 bp of microhomology between Sμ and the acceptor S region, i.e., one cannot discern whether the 1 bp came from Sμ or the acceptor S region, although this microhomology can increase up to 6 bp or more. In cells deficient in a NHEJ protein, e.g. KU or Ligase IV, or deficient in Mlh1 or Pms2, S-S junctions show further increased lengths of microhomology, especially if Sμ-Sα junctions are analyzed, as these S regions have the most homology with each other (99). To explain the increased lengths of junctional microhomology, investigators have thought that an alternate end-joining pathway, (A)-EJ or microhomology-mediated (MM)-EJ, substitutes for NHEJ, resulting in these longer microhomologies (100–102). However, it is not clear whether MM-EJ is really a defined pathway or instead that NHEJ has partially redundant components. For example, in the absence of Ligase IV, Ligase I and III can perform end-joining, but not as efficiently, and the other components of NHEJ can still participate (88, 103). Deficiencies in a NHEJ component might expose the DSBs to end-resection activities; also, the less efficient recombination might allow time for increased end-resection prior to ligation, and result in increased use of microhomology, which can help stabilize the junctions. In situations in which fewer S region DSBs are induced, e.g. in human patients heterozygous for AID with a C terminal deletion (104) or in *mlh1*$^{-/-}$ or *pms2*$^{-/-}$ mouse and human B cells (7, 99, 105), junctional microhomology is also increased. Perhaps this occurs because DSBs are limiting, thus delaying recombination. Note that although Msh2-deficient cells have reduced DSBs (7), they do not have increased junctional microhomology, indicating that reduced DSB frequency does not always result in increased use of microhomology. The explanation for this difference from Mlh1- and Pms2-deficient cells is unknown.

The AID C terminus is essential for CSR but not for SHM

The fact that AID lacking the C terminal 8–17 amino acids (ΔAID) cannot support CSR but appears to support normal SHM of V(D)J segments has been known for several years but the explanation is still unclear (106, 107). It is not due to aberrant targeting of AID to S regions, as cells expressing ΔAID appear to have approximately normal levels of Sμ and Sγ DSBs; thus, the problem is subsequent to break formation, despite the evidence that recruitment of UNG and Msh2-Msh6 to S regions is impaired in these cells (93, 108, 109). The DSB repair process appears to be aberrant, as there is reduced recruitment of NHEJ proteins to Sμ regions and increased lengths of S-S junctional microhomology in cells expressing ΔAID (93, 104, 110, 111). However, how the C terminus recruits NHEJ proteins, and if this is important for the greatly reduced CSR is still unknown.

The role of ATM during CSR and phosphorylation of AID

ATM kinase is a major regulator of the DNA damage response. ATM is rapidly activated by the binding of MRN to DSBs, and in turn, ATM phosphorylates MRN and several downstream effectors of DSB repair (112). Humans with ATM mutations have numerous problems due to poor DNA repair functions. Mouse splenic B cells lacking ATM have reduced CSR (30% of WT B cells) (113, 114), and impaired V(D)J recombination (115). In

response to S region DSBs, ATM induces phosphorylation of AID at S38 by an undefined pathway, and this increases the ability of AID to induce S region DSBs, and to bind to APE1 via an unknown protein (116). APE2 has not been tested. Thus, AID is activated by a feed-forward mechanism involving ATM. Phosphorylation of AID at S38 is not necessary for its deaminase activity in cell-free experiments, although activated cultured splenic B cells expressing AIDS38A have greatly reduced Sμ DSBs and CSR (116–118). It is hypothesized that this feed-forward mechanism increases AID activity at localized regions that have DSBs, such as at the IgH S regions, helping to explain why AID is so much more active at S regions than at off-target sites (91, 119, 120).

Interestingly, *atm*$^{-/-}$B cells induced to switch show decreased DSBs at Sγ regions, but have increased DSBs at Sμ(9). These data have been interpreted to suggest that the feed-forward activation of AID by ATM is very important for AID activity at acceptor S regions, and that when DSBs are poorly induced in the acceptor S region, Sμ DSBs accumulate due to lack of a downstream partner. These data also suggest that acceptor S region DSBs are limiting for CSR. It had previously been found that AID is more active at Sμ than at Sγ regions (37, 121), suggesting that AID attacks the Sμ region first (121). Taken together, these data lead to the hypothesis that ATM is activated by Sμ DSBs, resulting in phosphorylation of AID, which increases its activity at the downstream S region that is co-localized due to chromosome looping. The studies of chromosomal looping described above suggest that Sμ and acceptor S regions are located near each other prior to induction of AID and DSBs, as the looping might coincide with expression of GLTs. Thus, when AID is activated by phosphorylation, the acceptor S region might be localized sufficiently near to be attacked. Close localization of the two S regions also decreases the likelihood of aberrant recombination events. Surprisingly, ATM deficiency has no effect on cell cycle regulation of S region DSBs, as they are restricted to G1 phase in *atm*$^{-/-}$ cells, just as in WT cells, indicating that Sμ DSBs only accumulate during G1 phase and are repaired before S phase, even if the DSBs do not undergo Sμ-Sx recombination (9).

Function of 53BP1 in CSR

One of the DNA repair proteins that binds DSBs in response to the phosphorylation activities of ATM is 53BP1. CSR is reduced by 90% in cells deficient in 53BP1, but its roles in CSR are still not clear. ATM phosphorylates H2AX, converting it to γH2AX, which in turn interacts with 53BP1 (122), although additional kinases, other enzymes, and histone modifications also recruit and activate 53BP1 (123, 124). Resection at S region DSBs during G1 phase is normally inhibited by 53BP1 and its effector protein Rif1, two proteins very important for CSR that function epistatically, and whose activities favor recombination by NHEJ (120, 123, 125–131). Surprisingly, S-S junctions in *53bp1*$^{-/-}$ cells do not have increased microhomology (126). Another activity of 53BP1 essential for CSR is its ability to oligomerize (127, 132). 53BP1 likely binds DSBs in both Sμ and downstream S regions, and most likely the oligomerization of 53BP1 helps hold two different S regions together in the proper conformation. This might explain why inverted S region sequences and a segment from the IgH Eμ enhancer have been detected between the recombining S region segments in *53bp1*$^{-/-}$ cells (126). In addition, in *53bp1*$^{-/-}$ cells induced to undergo CSR there are numerous deletions within Sμ, suggesting that DSBs within Sμ failed to find an acceptor S

region recombination partner. Thus, 53BP1 is essential for CSR, and appears to have multiple roles, although they are still not entirely understood.

Questions remaining

There are many more interesting issues to discuss about CSR than can even be briefly discussed here due to space limitations. We recommend several recent reviews of CSR that include additional topics: (33, 49, 103, 133–135).

Although much is now known about CSR, there are still many questions left unanswered, some of which have been mentioned in this review. Although AID deaminates off-target genomic sites, and also induces DSBs at off-target sites, most of its activity is directed toward Ig loci. How AID is targeted to S regions is mostly unknown. We know that transcription, and probably RNA stalling and RNA splicing are important, but of course there are numerous transcribed genes with spliced transcripts and stalled Pol II. What causes the stalling of Pol II at S regions? S regions and S region transcripts are likely to bind specific proteins, for examples 14-3-3 (136) and PTBP2, that help recruit AID, but are there others? How are the looping of the C_H gene loci and enhancers regulated? What causes AID-induced S region DSBs to be restricted to G1 phase? Is AID activity restricted to G1 phase, as is UNG activity? What signaling mechanism causes the S region DSBs to be repaired prior to S phase even in the absence of ATM or p53(9)? What is the role APE2 in S region DSBs, and why isn't APE1 sufficient? Although APE1 is expressed as well as APE2 in splenic B cells induced to switch in culture, it is poorly expressed in germinal center B cells. Does the low expression of APE1 reduce CSR in germinal center cells? Is APE1 well-expressed during CSR in vivo in non-germinal center cells? How does phosphorylation of AID at S38 cause increased S region DSBs? Is this specific for S region DSBs? What does the C terminus of AID do during CSR? Although it might be involved in recruitment of NHEJ proteins, its deletion has a much greater effect on CSR than loss of NHEJ functions. What is the importance and what are the roles of histone modifications of IgH genes for CSR? What are the functions of AID expression in pro- and pre-B cells? is this advantageous or only deleterious?

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Abbreviations

AID	activation-induced cytidine deaminase
AP	apyrimidinic/apurinic
APE	AP endonuclease
ATM	ataxia telangiectasia mutant
BER	base excision repair
CSR	class switch recombination

DSB	double-strand break
NHEJ	non-homologous end-joining
SHM	somatic hypermutation
S region	switch region
ss	single-stranded
UNG	uracil DNA glycosylase
53BP1	p53-binding protein

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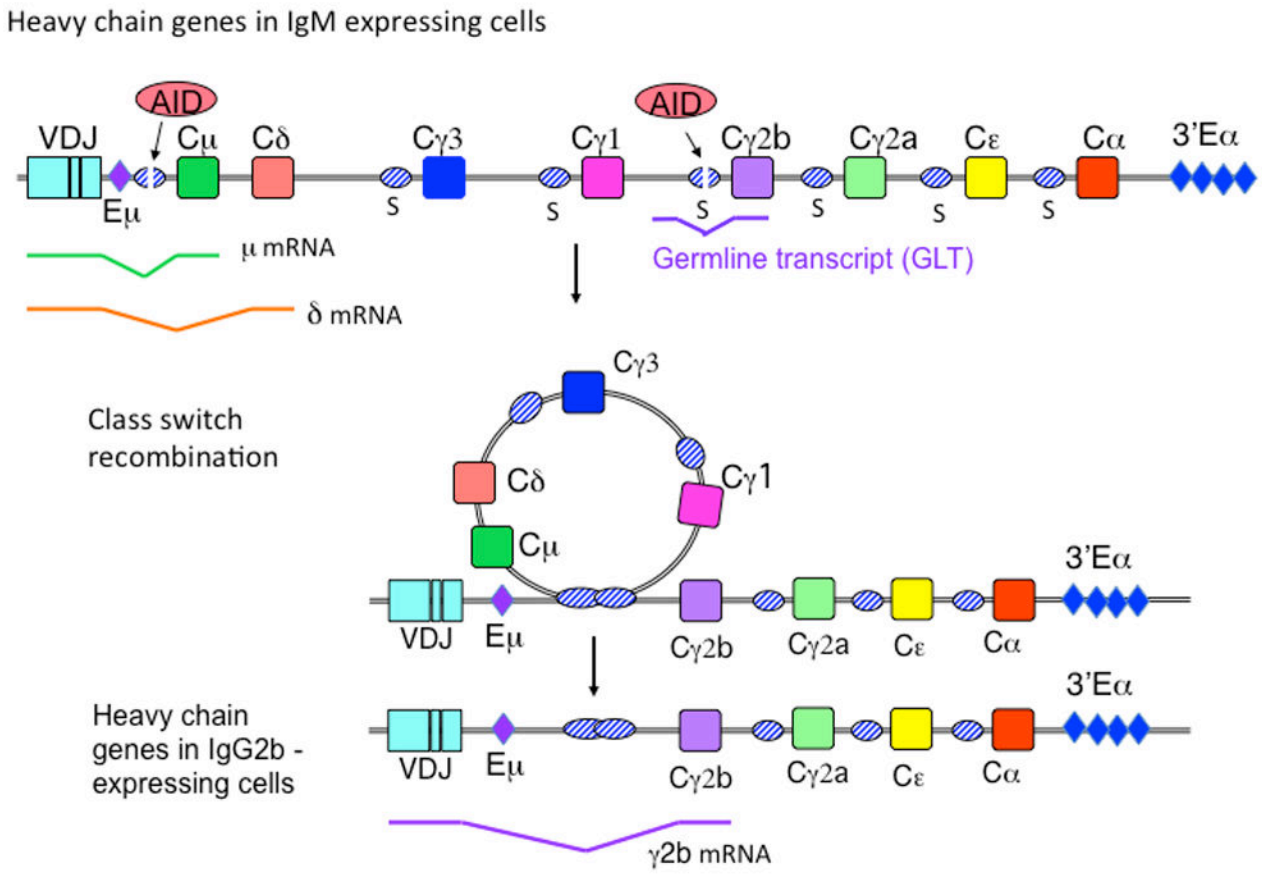

Figure 1. Diagram of the mouse IgH genes in naïve mature B cells expressing IgM and IgD, and CSR to IgG2b

During CSR to IgG2b, AID deaminates the Sμ and Sγ2b regions, instigating DSB formation. The Sμ and Sγ2b regions recombine by an intrachromosomal deletional recombination, which causes the expressed VDJ segment to become associated with the Cγ2b gene. Splicing diagrams of the μ and δ mRNAs, the γ2b germline transcripts (GLTs), and γ2b mRNA are indicated beneath the genes. Eμ and 3′Eα are the two major enhancers that regulate expression of Ig heavy chains and CSR.

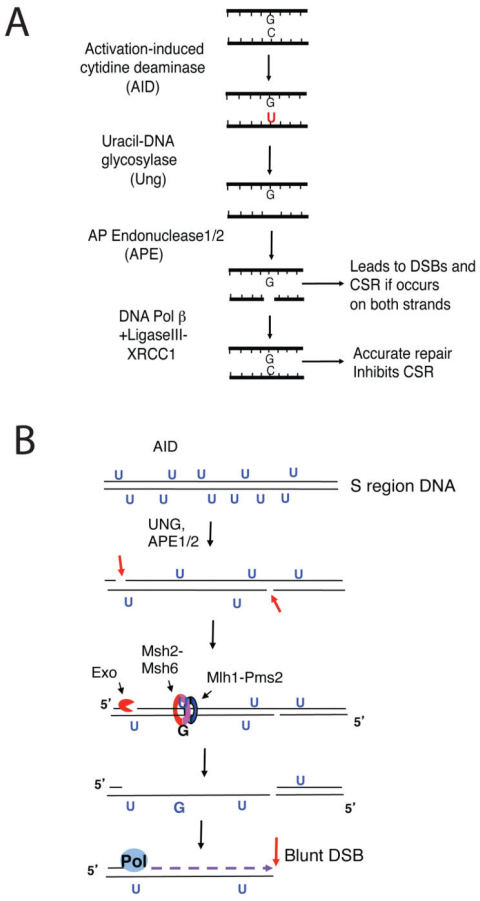

Figure 2. Models for the generation of DNA DSBs during CSR

(A) Diagram of how the base excision repair (BER) pathway converts AID-induced dUs to DNA breaks. (B) Diagram of a model for how the mismatch repair pathway converts SSBs produced by UNG and APE activity to DSBs appropriate for NHEJ. See text for more information.

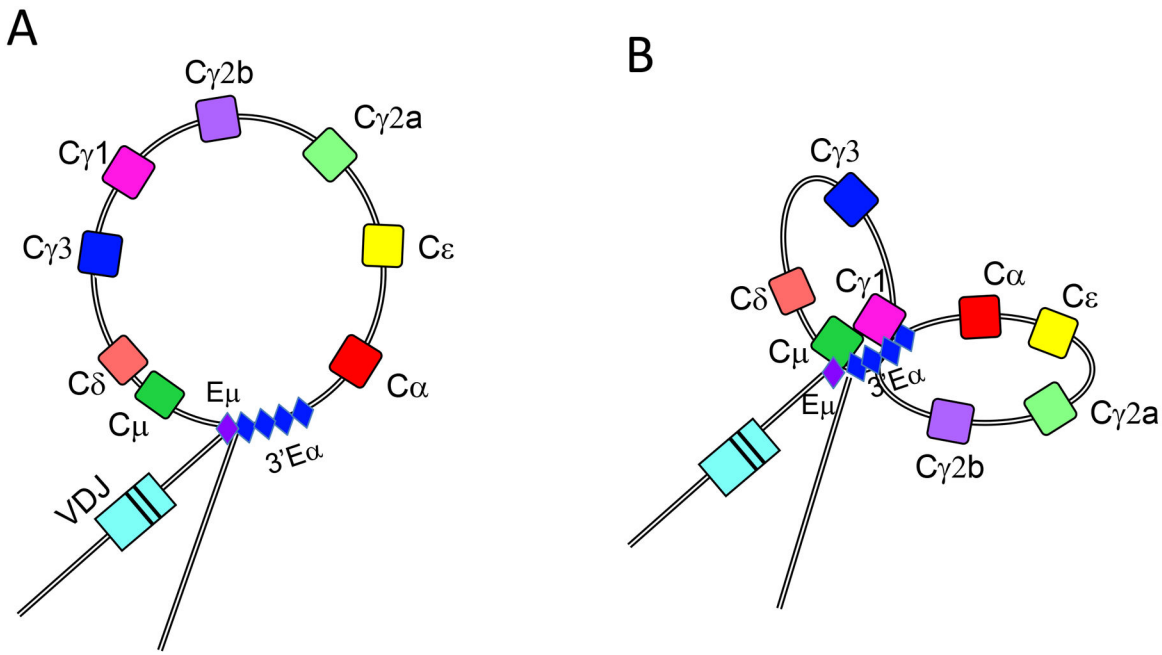

Figure 3. Diagrams of models of chromosome looping within the C_H gene locus obtained from chromosome conformation capture (3C) assays

(A) In mature naïve B cells the Eμ and 3′Eα enhancers are interacting. (B) A model for the interactions predicted from 3C assays for splenic B cells induced to switch to IgG1, showing interactions between the two enhancers, and the Em-Sm-Cμ, and Sg1-Cγ1 loci. The S regions are not diagrammed, and the sites of interaction are not precisely known, so the diagram only indicates approximate locations. See text for more explanation.