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Regulation of Immunoglobulin Class-Switch Recombination: Choreography of Noncoding Transcription, Targeted DNA Deamination, and Long-Range DNA Repair

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Abstract

Upon encountering antigens, mature IgM-positive B lymphocytes undergo class-switch recombination (CSR) wherein exons encoding the default C μ constant coding gene segment of the immunoglobulin (Ig) heavy-chain (*Igh*) locus are excised and replaced with a new constant gene segment (referred to as “C $_h$ genes”, e.g., C γ , C ϵ , or C α). The B cell thereby changes from expressing IgM to one producing IgG, IgE, or IgA, with each antibody isotype having a different effector function during an immune reaction. CSR is a DNA deletional-recombination reaction that proceeds through the generation of DNA double-strand breaks (DSBs) in repetitive switch (S) sequences preceding each C $_h$ gene and is completed by end-joining between donor S μ and acceptor S regions. CSR is a multistep reaction requiring transcription through S regions, the DNA cytidine deaminase AID, and the participation of several general DNA repair pathways including base excision repair, mismatch repair, and classical nonhomologous end-joining. In this review, we discuss our current understanding of how transcription through S regions generates substrates for AID-mediated deamination and how AID participates not only in the initiation of CSR but also in the conversion of deaminated residues into DSBs. Additionally, we review the multiple processes that regulate AID expression and facilitate its recruitment specifically to the Ig loci, and how deregulation of AID specificity leads to oncogenic translocations. Finally, we summarize recent data on the potential role of AID in the maintenance of the pluripotent stem cell state during epigenetic reprogramming.

1. OVERVIEW OF GENOMIC ALTERATIONS IN B CELLS

The aptitude of the vertebrate immune system to recognize a highly diverse set of antigens is achieved via adaptive immune lymphocytes called B and T cells. B cells are integral to the humoral immune response due to the expression of immunoglobulin (Ig) surface receptors that can be secreted as antibodies. Ig molecules are comprised of two heavy and two light chain polypeptides that are held together through disulphide bridges. The heavy and light

chains are comprised of a diverse amino-terminal variable region, responsible for antigen recognition and binding, and a carboxy-terminal constant region that determines effector function subsequent to antigen engagement. In humans and mice, three distinct genomic alterations create the enormous diversity of B cells and tailor them for the most efficacious immune response: V(D)J recombination, class-switch recombination (CSR), and somatic hypermutation (SHM). Some species such as sheep and chicken undergo a secondary diversification reaction termed gene conversion; this will not be discussed further here.

1.1. V(D)J recombination

In the fetal liver and adult bone marrow, developing B cells assemble the genes encoding the amino-terminal variable regions of the Ig heavy and light chains from component variable (V), diversity (D), and joining (J) “coding” gene segments through a somatic gene rearrangement process called V(D)J recombination (Schatz & Baltimore, 2004; Tonegawa, 1983). This reaction is initiated by the lymphocyte-specific endonuclease recombination activating genes 1 and 2 (RAG1/2) that generates DNA double-strand breaks (DSBs) at recombination signal sequences (RSSs) flanking the V, D, and J gene segments (Gellert, 2002; Oettinger, Schatz, Gorka, & Baltimore, 1990; Schatz, Oettinger, & Baltimore, et al., 1989). Components of the general classical nonhomologous end-joining (C-NHEJ) pathway joins DSBs between the gene segments to complete V(D)J recombination (Alt, Zhang, Meng, Guo, & Schwer, 2013; Boboila, Alt & Schwer, 2012; Schatz & Ji, 2011). In the *Igh* locus, the productive assembly of the recombined VDJ segment directly upstream of C μ generates the Ig μ polypeptide, which pairs with a similarly recombined κ or λ Ig light chain to generate an IgM molecule expressed on the surface of a mature naïve B cell. Alternative splicing of the VDJ exon to the C δ constant region segment allows for expression of IgD. The combinatorial usage of different V, D, and J segments combined with sequence modifications during V(D)J recombination allows the generation of a naïve B cell population expressing a highly varied repertoire of low-affinity surface IgM (or IgD) molecules (Alt et al., 2013; Boboila, Alt & Schwer, 2012; Schatz & Ji, 2011). These mature naïve B cells circulate through peripheral lymphoid organs, where upon encountering their cognate antigens in germinal centers (GC), they undergo two additional genomic alterations in the forms of CSR and SHM (Fig. 1.1). Both CSR and SHM require the activity activation-induced cytidine deaminase (AID) (Muramatsu et al., 2000; Revy et al., 2000).

1.2. Somatic hypermutation

During SHM, point mutations, and sometimes deletions and insertions, are introduced at a very high rate (10^{-2} to 10^{-3} per base pair per generation) into the recombined, expressed variable region of both the *Igh* and *Ig κ / λ* genes (McKean et al., 1984; Papavasiliou & Schatz, 2002; Peled et al., 2008; Rajewsky, 1996; Wagner & Neuberger, 1996). While mutations are found throughout the variable region exons, they are more frequently observed at RGYW/WRCY (R = A/G, Y = C/T, W = A/T) “hot spot” motifs (Rogozin & Diaz, 2004). Typically, the mutations accumulate within the complementarity determining region (CDR) loops, which generally reside in the Ig regions that contact antigen. SHM allows for the selection of a B cell with increased affinity for its cognate antigen. It should be noted that characterization of broadly neutralizing antibodies to HIV-1 in humans revealed, for the first time, that the framework regions that scaffold the CDR loops, and are normally thought to

be resistant and less tolerant of mutations, also accumulate SHM (Klein et al., 2013). As B cells accrue mutations, they are selected for their antigen-binding affinity, with those harboring mutations that enhance affinity receiving necessary survival signals to predominate the immune response.

SHM requires transcription through the expressed variable region exons, with mutations beginning from 100–200 bp downstream of the promoter and extending up to 1.5–2 kb further downstream, sparing the constant regions (Gearhart & Bogenhagen, 1983; Lebecque & Gearhart, 1990; Pech, Hocht, Schnell, & Zachau, 1981; Peters & Storb, 1996; Rada, Gonzalez-Fernandez, Jarvis, & Milstein, 1994; Rothenfluh, Blanden, & Steele, et al., 1995; Winter, Sattar, Mai, & Gearhart, 1997). In transgenic SHM substrates, the variable regions could be replaced with non-Ig sequences without loss of mutability, indicating that the primary nucleotide sequence does not contribute to the targeting of the mutational machinery (Michael et al., 2002; Peters & Storb, 1996; Tumas-Brundage & Manser, 1997; Yelamos et al., 1995). In a simplified model for SHM (Fig. 1.2), transcription through the variable region exons promotes the recruitment of AID, which deaminates deoxycytidines (dCs) to deoxyuridines (dUs) (Petersen-Mahrt, Harris, & Neuberger, et al., 2002). Replication across the dU:dG mismatch results in the introduction of transition mutations. On the other hand, removal of dU by the base excision repair (BER) enzyme uracil DNA glycosylase (UNG) prior to replication generates an abasic site, which when replicated across by error-prone DNA polymerases leads to the introduction of both transition and transversion mutations (Petersen-Mahrt et al., 2002). In addition, the dU:dG mismatch could be recognized by mismatch repair (MMR) proteins, which coupled with gap-filling by error-prone DNA polymerases can result in point mutations at A:T residues and/or short insertions and deletions throughout the variable region (Peled et al., 2008; Petersen-Mahrt et al., 2002).

Several excellent recent reviews have delved into the mechanism of SHM and should be consulted for a more in-depth treatise (Di Noia & Neuberger, 2007; Pavri & Nussenzweig, 2011; Saribasak & Gearhart, 2012; Teng & Papavasiliou, 2007). One aspect of SHM that merits brief discussion here is the mechanism by which AID, an exclusively single-stranded (ss) DNA-specific cytidine deaminase, accesses the variable region exons. One possibility is that transcription promotes negative supercoiling of DNA upstream of the elongating RNA polymerase, thus creating DNA topology conducive for AID binding (Kodgire, Mukkavar, Ratnam, Martin, & Storb, 2013; Shen & Storb, 2004). Another, not necessarily mutually exclusive, proposal is that the ssDNA binding protein replication protein A (RPA), which interacts with AID, stabilizes ssDNA bubbles within transcribed variable region exons, allowing AID-mediated deamination to facilitate SHM (Chaudhuri, Khuong, & Alt, et al., 2004). The differential requirement of RPA in CSR and SHM and its phosphorylation-dependent binding to AID will be addressed later.

1.3. Class-switch recombination

Immunoglobulin heavy-chain CSR exchanges the default C μ exons for an alternative set of downstream *Igh* C-region (C $_{\text{H}}$) exons, that is, C γ , C ϵ , or C α (Fig. 1.1). Thereby, the B cell is altered from expressing IgM to one producing a secondary antibody isotype such as IgG,

IgE, or IgA, respectively, with each antibody class having distinct effector functions during an immune response (Honjo, Kinoshita, & Muramatsu, et al., 2002; Stavnezer, Guikema, & Schrader, et al., 2008). CSR is a deletional-recombination reaction that occurs between repetitive DNA elements termed “switch” or S regions that precede each C_h gene.

According to the prevailing model for CSR, transcription through S regions allows generation of RNA:DNA hybrid structures, such as R-loops, revealing stretches of ssDNA that serve as substrates for AID (Chaudhuri & Alt, 2004; Chaudhuri et al., 2007; Yu & Lieber, 2003). Subsequent processing by components of the BER and MMR pathways convert the deaminated residues into DSBs. End-joining of DSBs between two S regions excises the intervening DNA sequence and completes CSR by placing a new constant gene directly downstream of the rearranged variable region exons. Thus, CSR allows for the generation of Ig molecules with the same affinity for the antigen, but with new effector functions (Boboila, Alt & Schwer, 2012; Stavnezer, 2011).

2. INITIATION OF CSR: S REGIONS AND GERMLINE TRANSCRIPTION

2.1. Requirement of S regions in CSR

The mouse *Igh* locus is comprised of eight C_h genes, each of which, except C δ , is preceded by a 1–12 kb long S region DNA element (Fig. 1.1). The human *Igh* locus is similarly organized, a notable exception being the presence of an uncharacterized $\sigma\delta$ repetitive sequence located between C μ and C δ that allows for the CSR-like exchange of IgM to IgD (Chen et al., 2009; Kluin et al., 1995). The primary sequences of S regions are not identical, but they do share similarities in that they are unusually G-rich on the template strand and consist of recurring motifs of varying lengths. S μ , for example, is approximately 3.2 kb long and is comprised of GAGCT pentameric motifs, with the AGCT palindromic sequence representing a canonical RGYW/WRCY sequence. S γ 1 is approximately 10 kb long and has multiple RGYW/WRCY sequences embedded within 49 bp repeat units. Other repeat motifs found in S regions include GGGGA/T in S μ , S γ 1, S γ 2b, and S γ 3 and GGGCT in S ϵ and S α . Most of the characterized switch junctions from cells that have undergone CSR fall within the S regions, thus it was logically postulated that S regions serve as recombination targets to drive CSR (Davis, Kim, & Hood, et al., 1980; Dunnick, Hertz, Scappino, & Gritzmacher, 1993; Dunnick, Rabbitts, & Milstein, et al., 1980; Kataoka, Yamawaki-Kataoka, Yamagishi, & Honjo, 1979; Nikaido, Nakai, & Honjo, et al., 1981; Obata et al., 1980; Sakano, Maki, Kurosawa, Roeder, & Tonegawa, 1980; Stavnezer, 1996; Takahashi et al., 1982; Zarrin, Goff, Senger, & Alt, 2008).

Unequivocal evidence for the requirement of S regions in CSR came from gene targeting studies. Deletion of S γ 1 completely abrogated CSR to IgG1 without affecting CSR to the other isotypes (Shinkura et al., 2003). Deletion of S μ also led to a severe defect in CSR to IgG1, as would be expected upon loss of the donor S region (Khamlichi et al., 2004; Luby, Schrader, Stavnezer, & Selsing, 2001). A more recent study, however, revealed that S μ deletion still allowed significant levels of CSR to IgG1, leading to the provocative proposal that in the absence of S μ , S γ 1 could serve as a donor S region (Zhang et al., 2010). A direct correlation between S region length and CSR frequency was shown, at least for S γ 1, when it was replaced with 49 bp S γ 1 repeats of varying lengths (Zarrin, Tian, Wang, Borjeson, &

Alt, 2005). This correlation is consistent with the difference in CSR frequency between IgG1 (12 kb S region) versus IgE (1 kb S region) observed in *ex vivo* stimulated splenic B cells. While IgG1 was the predominant antibody isotype following switching (~30% of total B cells), a remarkable increase in CSR to IgE (3% to >40% IgE) was observed after deletion of S γ 1 (Misaghi et al., 2010). The unique structural and/or sequence features of S regions are essential for CSR, as substitution of S γ 1 with non-S region sequence was unable to support CSR to IgG1, while replacing it with wildtype or synthetic S γ 2b sequences restored CSR to a level observed for a S γ 1 sequence of similar length (2 kb) (Zarrin et al., 2008). Taken together, these experiments provided strong evidence that S regions serve as targets for the recombination reaction.

2.2. Requirement of germline transcription

The molecular basis for the role of S regions is intricately linked to the essential requirement of “germline” transcription in CSR (Jung, Rajewsky, & Radbruch, et al., 1993; Zhang, Bottaro, Li, Stewart, & Alt, 1993). Individual C $_h$ genes (except C δ) are organized as transcription units comprised of a cytokine/activation-inducible promoter upstream of an intervening exon (I-exon), an intronic S region, and C $_h$ exons (Lennon & Perry, 1985) (Fig. 1.3). The primary transcript, initiating from the promoter upstream of the I-exon, proceeds through the S region and terminates downstream of the C $_h$ exons. Splicing of the primary transcript removes the intronic S region and joins the I-exon to the C $_h$ exons. The mature transcripts are polyadenylated, but do not code for any proteins, and are referred to as germline or sterile transcripts (Alt, Rosenberg, Casanova, Thomas, & Baltimore, 1982; Alt, Rosenberg, Enea, Siden, & Baltimore, 1982; Stavnezer-Nordgren & Sirlin, 1986; Yancopoulos et al., 1986). Transcription through individual C $_h$ genes induces CSR to that particular isotype (Snapper, Finkelman, & Paul, et al., 1988). For example, *ex vivo* activation of mouse splenic B cells with bacterial lipopolysaccharides (LPS) activates transcription through I γ 2b-S γ 2b-C γ 2b and I γ 3-S γ 3-C γ 3 transcription units and promotes CSR to IgG2b and IgG3, respectively. Conversely, addition of IL-4 inhibits transcription through γ 2b and γ 3 units and instead activates transcription through I γ 1-S γ 1-C γ 1 and I ϵ -S ϵ -C ϵ transcription units and CSR to IgG1 and IgE, respectively. Likewise, activation with TGF- β activates the I α promoter and allows for CSR to IgA (Lutzker, Rothman, Pollock, Coffman, & Alt, 1988; Rothman, Lutzker, Cook, Coffman, & Alt, 1988; Stavnezer et al., 1988). The μ promoter, on the other hand, drives constitutive transcription through S μ even in unstimulated B cells (Li et al., 1994). It is to be noted that *ex vivo* activation of mouse splenic B cells in culture with distinct combinations of cytokines/activators has been a convenient and well-established tool to elucidate CSR.

The strict correlation between induction of germline transcription and activation of CSR to particular isotypes provided a strong mechanistic link between the two processes. This notion was further validated through mutational analyses. Deletion of I-exon promoters or regulatory regions (3'-RR) at the 3'-end of *Igh* abolished or severely reduced CSR, while replacing the inducible I-exon promoters with heterologous constitutive promoters drove CSR in a cytokine-independent fashion (Bottaro et al., 1994; Cogne et al., 1994; Jung et al., 1993; Kuzin et al., 2000; Lorenz, Jung, & Radbruch, et al., 1995; Manis, van der Stoep, et al., 1998; Michaelson, Giannini, & Birshtein, et al., 1995; Pinaud et al., 2001; Qiu,

Harriman, & Stavnezer, et al., 1999; Seidl et al., 1998; Vincent-Fabert et al., 2010; Zhang et al., 1993). These studies thus provided experimental evidence for the notion that germline transcription of particular C_h genes renders them “accessible” for CSR (Stavnezer-Nordgren & Sirlin, 1986; Yancopoulos et al., 1986). We now know that important components of this accessibility include RNA polymerase II (Pol II)-dependent recruitment of AID to S regions (Pavri & Nussenzweig, 2011), transcription-coupled modifications of histones at S regions (Jeevan-Raj et al., 2011; Wang, Wuerffel, Feldman, Khamlichi, & Kenter, 2009), and generation of RNA:DNA hybrid structures that provide ssDNA substrates of AID (Chaudhuri et al., 2007).

Several intriguing reports have also suggested that germline switch transcripts might have mechanistic roles in CSR independent of transcription alone. Deletion of the $I\gamma 1$ -exon splice donor site to inhibit splicing of the primary switch transcripts specifically abrogated CSR to IgG1, even though transcription through $S\gamma 1$ was unaffected (Lorenz et al., 1995). Additionally, expression of $S\alpha$ transcripts from a plasmid enhanced CSR to IgA in a B lymphoma cell line (Muller, Giese, Henry, Mushinski, & Marcu, 1998). Collectively, the findings suggest that germline switch transcripts might have roles in the regulation of CSR, even though their mechanism of action is yet to be elucidated. We will thus focus on the well-characterized role of germline transcription in the generation of substrates compatible for AID-mediated deamination.

It has now been well documented that transcription through S regions *in vitro* and *in vivo* generates R-loops in which the template DNA strand is stably hybridized to the RNA while the nontemplate strand is looped out as ssDNA (Daniels & Lieber, 1995; Kao et al., 2013; Mizuta et al., 2003; Reaban & Griffin, 1990; Shinkura et al., 2003; Tian & Alt, 2000; Yu, Chedin, Hsieh, Wilson, & Lieber, 2003) (Fig. 1.4). The looped out nontemplate strand has also been proposed to assume additional structures such as stem-loops (Tashiro, Kinoshita, & Honjo, et al., 2001), or fourstranded G-quartets that are stabilized by Hoogsteen pairing between G-residues (Dempsey, Sun, Hanakahi, & Maizels, 1999; Sen & Gilbert, 1988). However, while R-loop formation (where the displaced template strand is single stranded) at transcribed S regions has been experimentally demonstrated in B cells undergoing CSR (Kao et al., 2013; Mizuta et al., 2003; Yu et al., 2003), there is no direct evidence that the ssDNA assumes additional secondary structures in switching B cells.

Formation of RNA:DNA hybrids at transcribed S regions is predicted from the primary sequence of S regions, given that the thermodynamic stability of G-rich RNA:C-rich DNA is much stronger than that of G-rich DNA:C-rich DNA (Ratmeyer, Vinayak, Zhong, Zon, & Wilson, 1994). This prediction is consistent with *in vitro* experiments where an S region transcribed in the physiological orientation forms stable R-loops, a structure not observed when the S region is transcribed in a nonphysiological orientation (G-rich template strand, C-rich RNA) (Daniels & Lieber, 1995; Mizuta et al., 2003; Reaban & Griffin, 1990; Shinkura et al., 2003; Tian & Alt, 2000). The physiological relevance of R-loops in CSR came from an elegant genetic study where a 1 kb synthetic S region with a G-rich nontemplate strand and the ability to form R-loops *in vitro* supported CSR *in vivo*; however, the same sequence transcribed in the non-R-loop forming reverse orientation was severely impaired at mediating CSR (Shinkura et al., 2003). The generally accepted role of R-loops

in CSR is that they provide ssDNA substrates for the key enzyme AID (Chaudhuri et al., 2007).

3. INDUCTION OF DNA LESIONS IN CSR: ESSENTIAL REQUIREMENT OF AID

3.1. Discovery of AID

The discovery of AID was a notable triumph that came at a time when the field was struggling to come to consensus on a cohesive model for the mechanisms of SHM and CSR. The discovery was facilitated by characterization of a B lymphoma line CH12F3 that can be induced to undergo CSR from IgM to IgA with high frequency (Nakamura et al., 1996). Using a PCRbased cDNA subtraction screen, AID was identified as a gene that was strongly induced when CH12F3 cells were stimulated to undergo CSR (Muramatsu et al., 1999). AID, encoded from the *Aicda* locus, is a protein of 198 amino acids (24 kDa) that is barely detectable in unstimulated CH12F3 cells, but increases in expression a remarkable 10-fold in 6 h following stimulation (Muramatsu et al., 1999). The essential role of AID in SHM and CSR came from two co-published studies. Generation of AID-deficient mice showed a striking and complete defect in both CSR and SHM (Muramatsu et al., 2000), while AID mutations in patients with an autosomal recessive form of hyper-IgM syndrome (HIGM2) resulted in a lack of SHM and CSR (Revy et al., 2000).

3.2. AID is a single-strand DNA deaminase

AID is one of 12 members of the APOBEC family of DNA/RNA cytidine deaminases. APOBEC1, the first to be discovered, is unique in its ability to selectively deaminate a specific C on a defined mRNA (Davidson & Shelness, 2000), while other members deaminate cytidines at multiple residues on ssDNA. *In vitro* biochemical studies demonstrated that AID is a ssDNA-specific cytidine deaminase, converting dCs to dUs, with no observable activity on double-strand DNA, RNA, or RNA:DNA hybrids (Chaudhuri et al., 2003; Dickerson, Market, Besmer, & Papavasiliou, 2003; Nabel, Lee, Wang, & Kohli, 2013; Pham, Bransteitter, Petruska, & Goodman, 2003; Ramiro, Stavropoulos, Jankovic, & Nussenzweig, 2003; Sohail, Klapacz, Samaranayake, Ullah, & Bhagwat, 2003; Yu, Roy, Bayramyan, Haworth, & Lieber, 2005). Based on bacterial cytidine deaminases that are homologous to APOBEC1 and AID, the deamination reaction proceeds via a direct nucleophilic attack at position 4 of the pyrimidine ring of cytosine by Zn^{2+} coordinated to AID. While X-ray crystallographic and NMR studies have revealed the structures of APOBEC family members (Vasudevan et al., 2013), the structure of AID/APOBEC proteins bound to nucleic acids have not been solved, leading to a dearth in our understanding of how AID engages its substrates. Still, *in vitro* assays identified an 11 amino acid hotspot recognition motif (residues 113–123), distinct from the active site residues (56–58 and 87–90) that confer specificity for the RGYW/WRCY hotspot motif. Replacing this recognition loop with one from another APOBEC family member (APOBEC3G or APOBEC3F) changed the mutation signature of AID toward that of the donor protein (Carpenter, Rajagurubandara, Wijesinghe, & Bhagwat, 2010; Pham, Calabrese, Park, & Goodman, 2011; Wang, Rada, & Neuberger, et al., 2010).

AID was shown to be a processive enzyme *in vitro*, catalyzing numerous cytidine deaminations on the same ssDNA fragment (Pham et al., 2003, 2011). However, AID is an inefficient enzyme, deaminating only about 3% of the cytidines even at preferred hotspot motifs. Mathematical formulation based on *in vitro* deamination data led to a “random walk” model wherein AID mainly traverses ssDNA by sliding in a bidirectional fashion, with a deamination probability of 1–7% per hotspot motif encounter (Mak, Pham, Afif, & Goodman, 2013; Pham et al., 2011). It has been suggested that the haphazard and inefficient activity of AID ensures that a wide variety of variable region mutations are tested for antigen affinity during SHM (Mak et al., 2013; Pham et al., 2011). How this inefficiency aids in CSR, where a high density of breaks is favored, is not clear at present. It is to be noted that the majority of *in vitro* studies have been carried out with bulky tags on AID, whose effect on the efficiency of the reaction has not been determined. Likewise, how the rate of deamination is altered by modifications in AID or its interactions with other proteins remains to be elucidated. Despite these unresolved issues that limit precise examination of its enzymatic activity, it is now unequivocally established that AID mediates CSR (and SHM) by acting as a ssDNA cytidine deaminase.

The ssDNA specificity of AID naturally leads to the question of how such substrates are generated during CSR. As discussed earlier, S regions, when transcribed *in vitro* in the physiological orientation, form R-loops in which cytidines in the displaced G-rich nontemplate strand can be efficiently deaminated by AID (Chaudhuri et al., 2003) (Fig. 1.4). A more direct correlation between R-loop formation and AID activity came from the observations that a G-rich DNA that supported CSR *in vivo* was efficiently deaminated by AID *in vitro* when the reaction was coupled to transcription, while a C-rich DNA that did not target CSR *in vivo* was a poor AID substrate *in vitro* (Chaudhuri et al., 2003). These observations led to a plausible and prevailing model wherein transcription through mammalian S regions generates ssDNA R-loop substrates to allow for the cytidine deaminase activity of AID. The deaminated DNA is subsequently processed into DSBs through the activities of BER and MMR proteins (Fig. 1.5). This R-loop-based model for AID access to S region DNA provides a convincing explanation for the essential requirements of S regions and S region transcription during CSR.

3.3. RNA editing by AID?

The sequence homology of AID with a *bona fide* RNA-editing enzyme, APOBEC1, led to the proposal that AID deaminates cytidines in specific mRNAs and that the “edited” mRNAs can then encode proteins required for CSR and SHM (Honjo, 2008). The requirement for *de novo* protein synthesis for DSB formation during CSR was argued to support this model (Begum et al., 2007, 2009; Begum, Kinoshita, Muramatsu, et al., 2004; Doi, Kinoshita, Ikegawa, Muramatsu, & Honjo, 2003), although an equally plausible explanation is that CSR requires labile proteins that function downstream of DNA deamination. In a variation of this mRNA-editing model, it was proposed that AID modifies microRNAs (miRNAs) that regulate expression of Topoisomerase I (Top1) (Kobayashi et al., 2009). According to this notion, AID modifies miRNAs that regulate Top1 mRNA, leading to a decrease in the amount of Top1 in cells undergoing CSR, which in turn alters DNA structure and induces cleavage in S regions (Kobayashi et al., 2009). However, the

mechanism by which Top1 levels can specifically induce DSBs in S regions remains unclear, and the identity of the RNA substrates of AID remain elusive. Furthermore, recombinant AID failed to deaminate cytidines in RNA (Nabel et al., 2013), even though one could argue that AID can only act on a very specific RNA moiety. Overall, at present, RNA-editing by AID remains a provocative idea. On the other hand, the observations that (a) uracils accumulate within S regions in an AID-dependent fashion (Maul et al., 2011), (b) deficiency in processing uridines in DNA severely impairs CSR (discussed below), and (c) AID associates with S regions *in vivo* (Nambu et al., 2003), provide compelling and overwhelming support for the idea that AID functions in CSR (and SHM) through direct DNA deamination, converting dCs to dUs at S regions.

4. PROCESSING OF DEAMINATED DNA: REQUIREMENTS FOR BER AND MMR PROTEINS

4.1. Removal of uracil residues from deaminated DNA

The detection of episomal circles comprised of the DNA excised between two S regions suggested that CSR proceeds through DSB intermediates (Iwasato, Shimizu, Honjo, & Yamagishi, 1990; Matsuoka, Yoshida, Maeda, Usuda, & Sakano, 1990). Additional evidence came from ligation-mediated PCR experiments that detected AID-dependent DSBs at S regions, and from the presence of phosphorylated H2AX (γ H2AX) foci, a well-characterized marker of DSBs, at the *Igh* locus (Petersen et al., 2001; Schrader, Guikema, Linehan, Selsing, & Stavnezer, 2007; Schrader, Linehan, Mochegova, Woodland, & Stavnezer, 2005; Wuerffel, Du, Thompson, & Kenter, 1997). Finally, the requirement for several DSB sensors and repair factors, including 53BP1, ATM, and components of the C-NHEJ pathway, strongly indicates that DSBs are generated during CSR (Alt et al., 2013). It is now generally believed that the BER and MMR pathways contribute to the conversion of deaminated cytidines into DSBs.

Components of the BER pathway play a major role in CSR. Through BER, the AID-introduced dU in S region DNA is removed by UNG to generate an abasic site (Fig. 1.5). The abasic site is recognized by the apurinic/apyrimidinic endonuclease APE1 generating a nick (Petersen-Mahrt et al., 2002). A closely spaced, similarly created nick on the opposite strand generates a staggered DSB. As predicted, mutations in UNG in humans and mice lead to impaired DSB formation at S regions and a severe block in CSR (Di Noia & Neuberger, 2002; Imai et al., 2003; Rada, Williams, et al., 2002; Schrader et al., 2005). Additionally, both mice heterozygous for APE1 deletion and CH12F3 cells with a homozygous deletion of APE1 have significantly impaired CSR (Guikema, Stavnezer, & Schrader, et al., 2010; Masani, Han, & Yu, et al., 2013; Schrader, Guikema, Wu, & Stavnezer, 2009). Overall, there is strong genetic data to support the proposed AID/UNG/APE1 pathway in CSR. In contrast to the well-established activities of UNG, it has been suggested that UNG might play a noncanonical role, since a UNG mutant deficient in uracil-removal activity can still promote CSR (Begum et al., 2007, 2009; Begum, Kinoshita, Kakazu, et al., 2004). A simpler, alternative explanation for this observation is that the residual activity in the mutant UNG protein is sufficient to remove uracils during CSR (Stivers, 2004).

During CSR, the dU:dG mismatch could also be processed via MMR (Rada, DiNoia, & Neuberger, et al., 2004). During this process, the Msh2–Msh6 heterodimer recognizes dU:dG mismatches, triggering the recruitment of adaptors and other effectors, such as the endonuclease heterodimer Pms2/Mlh1 and exonuclease 1 (Exo1), to generate nicks distal to the mismatch and subsequent conversion of the nicks into ssDNA gaps. Such activities occurring on opposite DNA strands could generate DSBs (Chaudhuri & Alt, 2004; Stavnezer et al., 2008). In mice, mutations in genes encoding MMR proteins (Msh2, Msh6, Mlh1, Pms2, or Exo1) significantly impair CSR, providing further support for this model (Bardwell et al., 2004; Ehrenstein & Neuberger, 1999; Ehrenstein, Rada, Jones, Milstein, & Neuberger, 2001; Roa et al., 2010; Schrader et al., 2004; Schrader, Vardo & Stavnezer, 2002, 2003).

4.2. Deamination of the template strand

Conversion of deaminated residues into DSBs during CSR requires AID activity on both DNA strands; however, purified AID, *in vitro*, could efficiently deaminate only the displaced single-stranded nontemplate strand, leaving the template strand largely untouched (Chaudhuri et al., 2003). Several proposals have been put forward to explain how template-strand deamination could be achieved. One possibility is that antisense transcription through S regions might allow AID to access both strands of the DNA (Perlot, Li, & Alt, et al., 2008). Additionally, AID has been shown to interact with components of the RNA exosome complex (Basu et al., 2011) and this interaction, perhaps in combination with RNaseH activity (Yu & Lieber, 2003), might remove the nascent transcript leading to R-loop collapse. In this model, the complementary DNA strands misalign due to the repetitive nature of S regions, resulting in exposed stretches of ssDNA on both DNA strands, allowing for AID access (Yu & Lieber, 2003). Finally, it has been proposed that negative supercoiling upstream of the elongating Pol II can allow access of AID to both strands of DNA (Kodgire et al., 2013; Shen & Storb, 2004). Thus, multiple processes could potentially allow for deamination of the template strand, but the precise mechanism is yet to be fully elucidated.

4.3. Conversion of ssDNA breaks into DSBs

Both BER and MMR have the potential to process deaminated DNA. However, it appears that CSR is more reliant on BER, given that UNG deficiency leads to a profound defect in CSR frequency, reducing it to less than 10% of wild-type levels (Di Noia & Neuberger, 2002; Imai et al., 2003; Rada et al., 2004). Residual CSR was completely abolished in B cells deficient for both UNG and Msh2 (or Msh6), suggesting that MMR plays a minor or back-up role in the initial processing of deaminated DNA (Rada et al., 2004; Shen, Tanaka, Bozek, Nicolae, & Storb, 2006). However, mutations in MMR genes (e.g., *Msh2*, *EXO1*) result in a nearly 30% defect in CSR, an effect greater than would be expected if MMR is acting as a mere back-up to the BER pathway in processing deaminated cytidines. It has been argued that MMR plays a role in the conversion of single-strand breaks (SSBs) on opposite DNA strands into DSBs (Stavnezer et al., 2008). If SSBs generated by AID–UNG–APE1 on opposite DNA strands are proximal to each other, they could be spontaneously converted into DSBs; however, if they are distal to each other, Msh2–Msh6 may bind to dU:dG mismatches that have not been processed by UNG and recruit EXO1 to excise DNA from the nearest 5'-SSB to the mismatch, creating a DSB with a 5'-overhang, which can

subsequently be filled in by a DNA polymerase (Stavnezer et al., 2008). Indeed, in B cells in which the tandem S μ repeats have been deleted, CSR is only reduced twofold, but is largely dependent on Msh2 and Mlh1, likely due to the requirement of the MMR proteins in conversion of distal SSBs that occur as a result of fewer AID targets (Min et al., 2003). Thus, while the MMR proteins could provide a complementary route to the major UNG-mediated processing of deaminated DNA, it is likely that the primary requirement of the MMR pathway in CSR lies in its ability to convert nicks and SSBs on opposite DNA strands into DSBs.

The majority of DSBs at S regions are staggered with 5' or 3' single-strand overhangs and must be processed into blunt or nearly blunt ends prior to ligation with another S region (Rush, Fugmann, & Schatz et al., 2004). Mre11, a component of the Mre11/RAD50/Nbs1 (MRN) complex, participates in CSR likely through its ability to process single-strand overhangs (Buis et al., 2008; Dinkelmann et al., 2009). Additionally, the structure-specific ERCC1–XPF endonuclease, which excises ssDNA tails at junctions with duplex DNA could participate in this process (Schrader et al., 2004; Tian & Alt, 2000). In contrast to excision or “chewing back” to form DSBs, S region breaks could be filled in by error-prone DNA polymerases, as these regions have been shown to accumulate mutations and deletions after B cell activation for CSR, much like mutations seen in variable region genes during SHM (Dunnick, Wilson, & Stavnezer, et al., 1989). This activity has been associated with polymerase *eta*, but could also be the result of additional error-prone DNA polymerases filling-in the 5'-overhangs left by DNA excision or partial end processing (Delbos et al., 2005; Dunnick et al., 1989; Faili et al., 2004; Schrader, Vardo & Stavnezer, 2003; Wilson et al., 2005; Zhou, Lottenbach, Barenkamp, & Reason, 2004).

Overall, the conversion of deaminated DNA into DSBs requires a large number of protein factors derived from multiple DNA repair pathways that have evolved to respond to general DNA damage. The mechanism through which this myriad of proteins comes together to effectively process the initial DNA lesion (deamination) without faithfully repairing the damage is an intriguing, unanswered question.

5. COMPLETION OF CSR: SYNAPSIS AND END-JOINING

The completion phase of CSR requires “synapsis” or close juxtaposition of the activated S regions followed by end-joining of DSBs between the participating S regions. The observation that the yeast I-SceI endonuclease can induce *Igh* class switching in B cells in which the S regions have been replaced with I-SceI sites strongly implicates the general cellular DNA-damage response and DSB-repair pathways in the synapsis and long-range end-joining of S region DSBs (Zarrin et al., 2007).

5.1. S region synapsis

Synapsis between S regions, which could be over a 100 kb apart, is critical for CSR. Unlike V(D)J recombination, where the RAG1/2 endonuclease efficiently cleaves RSSs only in the context of a synaptic complex, AID activity does not rely on prior interaction between two S regions, as constitutively transcribed S μ or S γ 2b regions integrated randomly into a pro-B cell line undergo a high rate of AID-induced internal deletions (Dudley et al., 2002). It has

been suggested that interactions between promoters that drive germline transcription and regulatory elements at the *Igh* locus ($E\mu$ located 5' of the $I\mu$ - $S\mu$ - $C\mu$ unit and $E\alpha$ located downstream of $C\alpha$) promote the formation of a three-dimensional genomic conformation that brings the S regions together in a S/S "synaptome" required for CSR (Ju et al., 2007; Kenter et al., 2012; Sellars, Reina-San-Martin, Kastner, & Chan, 2009; Wuerffel et al., 2007). In B cells activated to undergo CSR in culture, internal deletions within $S\mu$ are frequently observed even in cells that are still IgM-positive (Alt, Rosenberg, Casanova, et al., 1982; Gu, Zou, & Rajewsky, et al., 1993). These represent cells in which DSBs at $S\mu$ have not interacted, or synapsed, with DSBs at a distal S region, resulting in resolution through internal deletions in an intra-switch recombination reaction. Mutations in 53BP1 and H2AX lead to severe defects in CSR, but undergo internal $S\mu$ deletions at significantly higher frequency than wild-type B cells, suggesting that these proteins participate in the synapsis of S regions (Manis et al., 2004; Reina-San-Martin, Chen, Nussenzweig, & Nussenzweig, 2007; Ward et al., 2004). Recent studies have shown that 53BP1 acquires the ability to bind the newly identified protein Rif1 upon phosphorylation by ATM (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Feng, Fong, Wang, Wang, & Chen, 2013; Shi et al., 2013; Zimmermann, Lottersberger, Buonomo, Sfeir, & de Lange, 2013). It has been proposed that the interaction between 53BP1 and Rif1 is required for the protection of DSBs from 5'-3' end resection. In keeping with this notion, Rif1-deficient B cells are significantly impaired in undergoing CSR (Di Virgilio et al., 2013).

5.2. DNA end-joining

Mammalian cells harbor two major DNA repair pathways: homologous recombination (HR) and C-NHEJ. HR is the major pathway in postreplication repair and requires large stretches of homology, while C-NHEJ requires little or no homology, and operates throughout the cell cycle, but is the primary repair pathway in the G1 phase where HR is not active (Alt et al., 2013). CSR-associated DSBs are primarily observed in G1 phase cells and the recombining S regions do not have the extended homology required for HR; therefore, C-NHEJ is thought to be the major pathway in the joining of DSBs during CSR (Petersen et al., 2001; Schrader et al., 2007; Stavnezer et al., 2008). Indeed, mutations in components of the NHEJ pathway, including the DNA end-binding Ku70/Ku80 proteins, DNA ligase IV, or its cofactor XRCC4, lead to significant defects (20–40% of normal levels) in CSR (Boboila, Jankovic, et al., 2010; Boboila, Yan, et al., 2010; Casellas et al., 1998; Han & Yu, 2008; Manis, Gu, et al., 1998; Soulas-Sprauel et al., 2007; Yan et al., 2007).

Residual CSR in C-NHEJ-deficient B cells is mediated by microhomology-biased alternative end-joining (A-EJ), a poorly defined process that operates in the absence of C-NHEJ using a medley of factors, including XRCC1, Ligase III, Mre11, Parp1, and CtIP, from other DNA repair pathways (Dinkelmann et al., 2009; Lee-Theilen, Matthews, Kelly, Zheng, & Chaudhuri, 2011; Robert, Dantzer, & Reina-San-Martin, et al., 2009; Xie, Kwok, & Scully, et al., 2009). While there is a bias toward microhomology at S region junctions in C-NHEJ-deficient B cells, there is a substantial number of direct joins suggesting that microhomology is neither a signature nor a strict requirement for A-EJ (Alt et al., 2013; Boboila, Alt & Schwer, 2012; Boboila, Jankovic, et al., 2010; Boboila, Oksenyshyn, et al., 2012; Deriano & Roth, 2013; Lieber, 2010). While C-NHEJ (with A-EJ) plays a major role

in joining *Igh* DSBs during CSR, it is believed that HR repairs DSBs at non-*Ig* regions. This is evident from the observation that deficiency of the Rad51 paralog XRCC2, a key component of HR-mediated repair, leads to the generation of high levels of genome-wide AID-dependent DSBs (Hasham et al., 2010, 2012).

End-joining of S region DSBs in the absence of C-NHEJ to mediate CSR unmasks a significant mechanistic difference between CSR and V(D)J recombination, since the latter is entirely reliant on C-NHEJ. This likely reflects the ability of RAG1/2 to stably bind RSSs and actively shepherd DSBs to the NHEJ pathway (Lee, Neiditch, Salus, & Roth, 2004). Even though AID likely recruits proteins that participate downstream of DNA deamination (discussed below), it has not been shown to enforce engagement of C-NHEJ in the repair of DSBs at S regions.

6. AID PHOSPHORYLATION AT SERINE-38 AND A ROLE BEYOND DNA DEAMINATION

Mutations at the C-terminus of AID or deletion of the last 10 amino acids lead to a severe block in CSR without affecting AID deamination activity or binding to the S μ region (Barreto, Reina-San-Martin, Ramiro, McBride, & Nussenzweig, 2003; Ta et al., 2003). These observations suggested that the C-terminus of AID might participate in recruiting factors specific to CSR, such as those required for S region synapsis of endjoining downstream of DNA deamination. In keeping with this notion, AID lacking the C-terminus, unlike the wild-type protein, does not interact with DNA-PKcs (Wu, Gerald, Platt, & Cascalho, 2005). However, DNAPKcs-deficient cells show significant levels of CSR (Franco et al., 2008). Additionally, B cells expressing the mutant AID protein are inefficient in recruiting UNG and Msh2–Msh6 to S regions during CSR (Ranjit et al., 2011). Conflictingly, mutations at S μ , which are dependent on UNG and Msh2–Msh6, occur at normal levels in cells expressing the mutant protein (Barreto et al., 2003). Overall, it is still not clear how the C-terminus of AID participates in CSR. The role of AID in steps downstream of deamination, however, has been extensively studied in the context of AID phosphorylation and is discussed below.

6.1. Role of AID phosphorylated at Serine-38 in DSB formation

Purification of AID from B cells undergoing CSR followed by mass spectrometric analyses demonstrated that AID is phosphorylated at serine residue-38 (S38) (Basu et al., 2005; McBride et al., 2006). The S38 residue lies within a protein kinase A (PKA) consensus phosphorylation site and can be phosphorylated *in vitro* and *in vivo* by PKA (Basu et al., 2005; McBride et al., 2006; Pasqualucci, Kitaura, Gu, & Dalla-Favera, 2006; Vuong et al., 2009). The association of PKA with transcribed S regions undergoing CSR strongly suggested that PKA-mediated AID phosphorylation occurs at S regions (Vuong et al., 2009).

B cells from mice with an S38A knock-in mutation (*AID*^{S38A}) are substantially impaired in mediating CSR, indicating that AID phosphorylated at S38 (pS38-AID) plays a critical role in CSR (Cheng et al., 2009; McBride et al., 2008). *AID*^{S38A} is as active as wild-type AID in its ability to deaminate ssDNA and to bind S region DNA, indicating that neither the S38

residue nor its phosphorylation is required for DNA enzymatic activity on ssDNA or association with S regions (Basu et al., 2005; Vuong et al., 2009). However, *AID*^{S38A} B cells are impaired in their ability to generate DSBs at S regions, leading to the suggestion that this specific phosphorylation event might be required at a step downstream of DNA deamination (Vuong et al., 2013). Indeed, immunoprecipitation experiments demonstrated that pS38-AID interacts with APE1, the enzyme critical for the generation of DNA breaks downstream of AID and UNG activities during CSR (Vuong et al., 2013) (Fig. 1.6). Taken together, these observations provide strong evidence that phosphorylation of AID at S38 promotes its ability to interact with APE1 and facilitate formation of DSBs during CSR. The interaction between AID and APE1 is not direct and proteins that mediate this interaction have not yet been described.

Attempts to understand the mechanism by which AID phosphorylation is initially induced led to the striking observation that a catalytically inactive mutant of AID bound to S regions was not efficiently phosphorylated and failed to interact with APE1, suggesting that DNA lesions activate AID phosphorylation (Vuong et al., 2013). Consistent with this idea, AID phosphorylation and APE1 interaction are significantly subdued in B cells deficient for both UNG and Msh2 where DSBs are not induced. Treating UNG–Msh2 mutant B cells or cells expressing catalytically inactive AID with ionizing radiation to induce genome-wide DSBs restored both AID phosphorylation and APE1 binding. Thus, DSBs induce AID phosphorylation at S38.

The above findings elicit the question of what senses DSBs to induce AID phosphorylation. A primary participant in the general cellular response to DSBs is ATM, a kinase that phosphorylates a vast number of substrates required for responding to and repairing DSBs (Bensimon, Aebersold, & Shiloh, et al., 2011). B cells lacking ATM are severely impaired in their ability to undergo CSR, and this defect was attributed primarily to a failure to react to and repair DSBs (Lumsden et al., 2004; Reina-San-Martin, Chen, Nussenzweig, & Nussenzweig, 2004). Assessing AID phosphorylation in ATM-deficient cells demonstrated that the phosphorylation-dependent interaction between AID and APE1 was significantly subdued, thereby unmasking a novel role of ATM in inducing DSBs (Vuong et al., 2013). Thus, a single protein, ATM, regulates both the generation and repair of DSBs during CSR, thereby coordinating the DNA-damage response with DNA repair. It is to be noted that while ATM is required for efficient AID phosphorylation, AID is not thought to be a direct target of ATM kinase; rather, PKA is likely the direct AID kinase (Vuong et al., 2009). The mechanism through which ATM activation is transduced into PKA activation remains unclear.

6.2. Positive feedback loop in amplifying DSBs

The observation that DSBs were both dependent on and required for AID phosphorylation led to the proposal that a positive feedback loop amplifies DNA breaks at S regions through AID phosphorylation (Fig. 1.6). Envisioning a likely scenario where a high density of DSBs promotes end-joining between DSBs generated in two distal S regions necessitates a process of DNA break amplification for efficient CSR. A positive feedback loop provides a model whereby even after assembly of AID and PKA at S regions (Vuong et al., 2009), AID will

not be efficiently phosphorylated until a DNA break is generated. Once a DNA break is formed, the rapid activation of AID phosphorylation and DSB formation will result in the synchronous activation of many molecules of AID bound to an S region. The high density of DSBs in S regions thus generates many broken DNA ends that promote the ligation of distal DSBs, thereby subverting normal DNA repair. When AID phosphorylation is blocked, as in *AID^{S38A}* B cells or reduced, as in PKA hypomorphic mutant B cells, the low density of DSBs induced at individual S regions could be resolved as inefficient CSR (Vuong et al., 2009). Thus, while APE1 can passively access the abasic sites generated through basal AID activity, its active recruitment to the sites of AID-induced deamination through its interaction with pS38-AID would facilitate the rapid and efficient conversion of abasic sites into DNA breaks.

A DSB-inducing positive feedback loop requires coordinate recruitment of both AID and PKA to recombining S regions, which may be a regulatory mechanism to limit AID activity at non-*Ig* genes. While AID can bind and deaminate several non-*Ig* genes, without concomitant PKA binding very few of these lesions would be converted into DSBs in the absence of AID phosphorylation. Thus, the two-tiered mode of AID activation (recruitment to S regions and subsequent phosphorylation by PKA) provides a mechanism to generate a high density of DSBs specifically at *Igh* S regions during CSR while restricting DSB formation at non-*Ig* sites (Fig. 1.6).

If ATM is critical for the generation of a high density of DSBs in S regions, how does one then explain the increased DSBs and chromosomal translocations in ATM-deficient B cells (Franco et al., 2006)? One possibility is that ATM forces CSR to proceed through the canonical BER pathway, the major effector of CSR (Vuong et al., 2013). If true, ATM-deficient cells would not actively engage BER proteins, especially APE1, to process the dU:dG mismatches; rather, the MMR proteins would convert the deaminated residues into DNA breaks. ATM-deficient B cells are impaired in inter-, but not intra-S region recombination, indicating reduced synapsis of DSBs at S regions (Reina-San-Martin et al., 2004). This defect, when combined with a failure to enforce cell-cycle checkpoints at the G1-S transition, could explain the persistence of DSBs that can partake in aberrant chromosomal translocations (Franco et al., 2006). Therefore, the AID–APE1–DSB–ATM loop represents a positive feedback mechanism to generate a threshold number of DNA breaks precisely within transcribed S regions that not only promotes the joining of distal S regions to drive successful CSR, but also suppresses oncogenic translocations.

6.3. Role of AID in recruiting RPA to S regions

In addition to interacting with APE1, pS38-AID also gains the ability to interact with the ssDNA binding protein RPA. The phosphorylation-dependent interaction between AID and RPA was discovered in biochemical assays to identify factors that allowed AID to deaminate variable region genes, which do not form R-loops upon transcription (Basu et al., 2005; Basu, Wang, & Alt, et al., 2008; Chaudhuri et al., 2004). Thus, it was reasonable to propose that the pS38-AID_RPA complex, by virtue of the ssDNA binding ability of RPA, stabilizes ssDNA within transcription bubbles at variable region genes, thereby generating AID substrates during SHM (Chaudhuri et al., 2004). Consistent with this notion, B cells

from *AID*^{S38A} mice were substantially impaired in mediating SHM (Cheng et al., 2009; McBride et al., 2008). The defect was particularly pronounced in mice haploinsufficient for *AID*^{S38A} where the frequency of SHM was reduced to near background levels. Additionally, *Xenopus laevis* S regions, which are A:T rich and do not form R-loops, could still mediate CSR in mouse B cells by deaminating AGCT sequences in a pS38-AID_RPA complex-dependent SHM-like reaction (Zarrin et al., 2004). Taken together, these observations have led to the model that pS38-AID, through its ability to bind RPA, is critical for the recruitment of AID to variable region genes during SHM (Chaudhuri et al., 2004).

While identified as an AID interactor to facilitate SHM, RPA was also shown to bind S regions in a pS38-AID-dependent fashion (Chaudhuri et al., 2004; Vuong et al., 2009; Yamane et al., 2011). The CSR defect in *AID*^{S38A} B cells could thus be a combination of a failure of AID to recruit both APE1 and RPA to S region DNA. The observation that second-site mutations in *AID*^{S38A} (*AID*^{S38AT40D}) restores RPA binding to AID in the absence of phosphorylation, and partially rescues the CSR defect of *AID*^{S38A} protein, suggests that RPA plays a direct role in the recombination reaction (Basu et al., 2008). The role of RPA in CSR has not been fully elucidated. RPA bound to S regions may function downstream of deamination to recruit UNG or MMR proteins, which convert deaminated cytidines to DSBs. Additionally, RPA might recruit proteins such as 53BP1 and H2AX to DSBs to promote synapsis of DSBs between distal S regions. The known requirements of UNG, MMR proteins, 53BP1, and H2AX in CSR, and the reported interactions of these proteins with RPA, support a role for RPA downstream of DNA deamination (Vuong & Chaudhuri, 2012). It is also possible that RPA does not participate directly in the recombination reaction, but rather is required in the salvage of unrepaired AID-instigated DSBs at *Ig* and non-*Ig* genes by homology-based pathways (Yamane et al., 2013). A formal demonstration of the requirement of RPA in recruiting AID to variable region genes during SHM and defining the role of RPA in CSR remain outstanding issues.

7. MULTIFACETED REGULATION OF AID EXPRESSION AND ACTIVITY

AID is a general mutator and it comes as no surprise that it is regulated at multiple levels, including almost exclusive activated B cell-specific expression, miRNA-mediated regulation of *Aicda* mRNA stability, subcellular localization, phosphorylation, and specific recruitment to the *Ig* locus (Fig. 1.7). Below we outline our current understanding of the regulatory mechanisms that restrain AID activity.

7.1. Transcriptional regulation

AID is expressed primarily, though not exclusively, in activated B cells in GCs and can be induced in mature B cells activated *ex vivo* with cytokines or bacterial LPS through a combination of transcriptional activators and repressors (Lee-Theilen & Chaudhuri, 2010). The *Aicda* locus, which encodes AID (Muramatsu et al., 2000), contains four distinct regions of transcription factor-binding sites, with two regions lying upstream of the transcription start site (TSS), one within the first intron and the final residing approximately 17 kb downstream of the promoter (Crouch et al., 2007; Tran et al., 2010; Yadav et al., 2006) (Fig. 1.8).

Region 4 (RIV), lying ~8 kb upstream of the promoter, contains binding motifs for Stat6, Smad3/4, and NF- κ B, which are expressed in response to IL-4, TGF- β , and CD40 ligation, respectively (Huong le et al., 2013; Tran et al., 2010). Two C/EBP binding sites located within this region were shown to be indispensable for the enhancer activity of RIV on AID expression in activated B cells (Huong le et al., 2013; Tran et al., 2010). In addition, c-Myc, has been shown to be required for normal CSR due, in part, to its transcriptional induction of AID gene expression via binding to the RIV regulatory region (Dominguez-Sola et al., 2012; Fernandez et al., 2013).

Region 1 (RI), located immediately upstream of the TSS, contains binding sites for NF- κ B (p50), Stat6, HoxC4, and Pax5 (Park et al., 2009; Yadav et al., 2006). Signaling via IL-4 and CD40 ligation were shown to synergize and induce Stat6 and NF- κ B binding, respectively, to RI, while mice deficient in Stat6 or p50 exhibited impaired AID expression in response to these stimuli (Dedeoglu, Horwitz, Chaudhuri, Alt, & Geha, 2004). HoxC4, expressed in GC B cells upon CD40 engagement and cytokine stimulation, induces AID expression through binding a highly conserved site at the *Aicda* promoter (Park et al., 2009, 2013).

The intronic regulatory region 2 (RII) contains sites for both activating (Pax5 and E-proteins) and inhibitory (c-Myb and E2F) proteins (Gonda et al., 2003; Huong le et al., 2013; Sayegh, Quong, Agata, & Murre, 2003; Tran et al., 2010). It has been proposed that B-cell-specific induction of the *Aicda* gene is achieved by the expression of Pax5 and E-proteins upon B cell activation (Gonda et al., 2003; Sayegh et al., 2003; Tran et al., 2010). Both c-Myb and E2F are factors expressed in naïve and non-B cells, and it is thought that the inhibition of *Aicda* promoter activation by the binding of these factors may either be blocked, perhaps by Pax5 and E-protein binding, or overcome by cytokine-induced stimulating transcription factors (Huong le et al., 2013; Tran et al., 2010). Ectopic expression of Pax5 was shown to be sufficient to induce *Aicda* expression in a plasmacytoma cell line in the absence of additional stimuli that activate NF- κ B expression (Dege & Hagman, 2013). In addition to the binding of c-Myb and E2F to RII, it is believed that active inhibition of *Aicda* gene transcription is achieved via the antagonistic activity of inhibitor of differentiation (Id) proteins (Gonda et al., 2003; Sayegh et al., 2003). Id proteins, which lack a DNA-binding domain, inhibit transcription of target genes by forming heterodimers with activating transcription factors such as E2A. Both Id2 and Id3 have been implicated in forming heterodimers with Pax5 and E-proteins, respectively, and impair their binding to RII of the *Aicda* locus (Gonda et al., 2003; Sayegh et al., 2003).

Furthest from the TSS is region 3 (RIII), with a BATF binding site that is believed to act as an enhancer element, as *Batf* deletion leads to abrogation of AID expression and CSR (Betz et al., 2010; Crouch et al., 2007; Ise et al., 2011). Interestingly, a recent study employing an *Aicda* locus transgenic mouse system has shown that deletion of both RII and RIV abrogates normal *Aicda* expression in activated B cells; thus the presence of RIII cannot compensate for the loss of these two regions in inducing AID expression (Huong le et al., 2013).

The sex hormones estrogen and progesterone have also been associated with the modulation of AID expression in murine splenic B cells activated to undergo CSR (Mai et al., 2010; Pauklin & Petersen-Mahrt, 2009; Pauklin, Sernandez, Bachmann, Ramiro, & Petersen-

Mahrt, 2009). Estrogen-bound estrogen receptor induced AID transcription either by directly binding and activating the AID promoter, or indirectly by binding and activating the *HoxC4* gene (Mai et al., 2010; Pauklin & Petersen-Mahrt, 2009; Pauklin et al., 2009). In contrast, progesterone-bound progesterone receptor reportedly inhibits transcription of the *Aicda* locus by binding around the RI region upstream of the promoter (Pauklin & Petersen-Mahrt, 2009). The regulation of AID by hormonal factors could have implications in hormone-based oncogenesis and autoimmunity; however, their importance in the normal CSR mechanism remains to be elucidated (Incorvaia, Sicouri, Petersen-Mahrt, & Schmitz, 2013; Maul & Gearhart, 2009; Pauklin & Petersen-Mahrt, 2009; Pauklin et al., 2009).

7.2. Posttranscriptional control of *Aicda* mRNA

The binding of small RNA species or miRNAs to a target mRNA sequence results in mRNA silencing by translational repression and mRNA degradation. Several miRNAs, including miR-155, miR-181b, and miR-361, have been implicated in regulating the vigor of AID mRNA by binding to its 3'-untranslated region (3'-UTR) (Borchert, Holton, & Larson, et al., 2011; de Yebenes et al., 2008; Dorsett et al., 2008; Teng et al., 2008). In addition to playing a role in general GC structure and function, miR-155 was found to be upregulated in murine B cells undergoing CSR (Teng et al., 2008; Thai et al., 2007). Disruption of the miR-155 binding site within the 3'-UTR of AID mRNA led to increased AID expression, CSR, and *c-Myc:Igh* translocations (Dorsett et al., 2008; Teng et al., 2008). Interestingly, the transcriptional repressor Bcl6, whose expression is integral for GC formation and development, regulates miR-155 expression. Bcl6 binds to the promoter of miR-155, as well as to the intronic region upstream of the miR-155 sequence and represses miR-155 expression (Basso & Dalla-Favera, 2012). A second miRNA sequence shown to be a target for Bcl6 binding and repression, miR-361, was also identified as an AID 3'-UTR binding miRNA (Basso & Dalla-Favera, 2012). The role, if any, of miR-361 in the regulation of AID mRNA stability during the GC reaction and/or CSR remains to be determined. Finally, the expression of miR-181b in activated murine B cells impairs CSR efficiency and leads to decreased AID mRNA and protein levels (de Yebenes et al., 2008).

7.3. Compartmentalization of AID activity

Regulating subcellular localization of proteins is regularly employed by cells to sequester enzymes away from their substrates. Not surprisingly, this strategy is employed with AID, which is predominately cytoplasmic in the B cell and thus limited in its access to its DNA substrate in the nucleus (Ito et al., 2004; Pasqualucci et al., 2004; Rada, Jarvis & Milstein, 2002). This is achieved, in part, by active export via its C-terminal nuclear export signal (NES), which mediates exportin1-dependent nuclear export (Brar, Watson, & Diaz, et al., 2004; Ito et al., 2004; McBride, Barreto, Ramiro, Stavropoulos, & Nussenzweig, 2004). In addition, nuclear exclusion by a "cytoplasmic retention determinant" also present in the C-terminus of AID, but distinct from the NES, has been implicated in the cytoplasmic sequestering of AID (Patenaude et al., 2009). It is of note that the NES region overlaps with the C-terminal amino acid residues shown to be indispensable for CSR, but that are not required for SHM (Barreto et al., 2003; Ta et al., 2003).

In addition to a C-terminal NES, AID contains a bipartite nuclear localization signal (NLS) at its N-terminal domain. While, there are conflicting reports regarding the requirement of the NLS in the import of AID into the nucleus (Brar et al., 2004; Hu et al., 2013; Patenaude et al., 2009), it has been demonstrated that positively charged residues throughout the protein sequence, in both the N and C-terminal regions, come together to mediate nuclear transport (Hu et al., 2013). Thus, AID can be shuttled between the nucleus and cytoplasm in an unbalanced, cytoplasmic-dominant manner. AID was recently found to accumulate in subnuclear nucleolar structures, which are hubs for RNA metabolism (Hu et al., 2013). Mutations that abrogated AID localization to these structures resulted in reduced levels of CSR (Hu et al., 2013). Whether subnuclear compartmentalization of AID is important for its interaction with potential targeting factors or for the regulation of AID activity by further sequestration remains an open question.

AID that does make its way into the nucleus has been shown to have a short half-life due to destabilization via polyubiquitination (Aoufouchi et al., 2008). The importance of degrading nuclear AID was underscored when proteasome inhibition resulted in nuclear-restricted AID unleashing enhanced mutagenicity within the *Ig* loci and throughout the genome (Aoufouchi et al., 2008). A ubiquitin-independent mechanism to degrade nuclear AID involving REG γ , a proteasome activating protein, has also been reported (Uchimura, Barton, Rada, & Neuberger, 2011). Splenic B cells from REG γ -deficient mice underwent higher levels of CSR, as compared to littermate controls (Uchimura et al., 2011). Therefore, regulation of the ability of AID to enter and remain in the nucleus modulates its mutational potential. However, sequences in AID that regulate its residence in the nucleus overlap with residues required for its CSR or SHM activities, thereby complicating interpretation of mutational analysis.

7.4. AID phosphorylation

In addition to S38, AID is phosphorylated at several other residues—serine 3 (S3), threonine 27 (T27), T140, and tyrosine-184 (Y184) (Basu et al., 2005; Chaudhuri et al., 2004; Gazumyan et al., 2011; McBride et al., 2006, 2008; Pasqualucci et al., 2006) (Fig. 1.7). The S3 residue was identified as a site on recombinant AID that could be phosphorylated *in vitro* by protein kinase C (PKC) (Gazumyan et al., 2011). Mutation of S3 to alanine (AID^{S3A}) does not affect the DNA deaminase activity of AID (Gazumyan et al., 2011); however, expression of AID^{S3A} in AID-deficient B cells and fibroblasts increases CSR and SHM, suggesting that phosphorylation of S3 is an inactivating event. Consistent with this notion, expression of the mutant protein significantly increased *c-Myc:Igh* translocation frequency over that induced by the wild-type AID protein (Gazumyan et al., 2011). The precise mechanism through which phosphorylation at S3 regulates AID function is yet unknown.

Mass spectrometric analysis of AID purified from B cells identified T140 and Y184 as phosphorylated residues (Basu et al., 2005; McBride et al., 2006, 2008). Mutation of T140 to alanine in AID (AID^{T140A}) does not affect DNA deaminase activity (McBride et al., 2008). While B cells from mice expressing AID^{T140A} displayed only a mild defect in CSR, a more profound defect in SHM was observed (McBride et al., 2008). The mechanism by which T140 phosphorylation modulates AID activity during SHM is not known, but it is

likely that this phosphorylation event modulates the ability of AID to interact with an unidentified factor(s) that is required for SHM but not CSR. Mutation of Y184 to alanine does not affect the ability of AID to mediate CSR (Basu et al., 2005). T27 was identified as a residue phosphorylated by PKA, and mutation of T27 to alanine severely impairs CSR without affecting the ssDNA deaminase activity of AID (Basu et al., 2005; Demorest, Li, & Harris, 2011; Pasqualucci et al., 2006). However, it is not clear if the T27 residue is phosphorylated *in vivo* and how it influences CSR. Additional phosphorylation sites at S41 and S43 were detected in Sf9 cells expressing AID, but whether these residues are functionally relevant during CSR or SHM is not known (Pham et al., 2008). In general, none of the currently identified AID phosphorylation events regulates its DNA deaminase activity; rather, they appear to mediate the interaction of AID with other proteins to modulate CSR and/or SHM.

8. TARGETING OF AID TO THE IG LOCI

The specific recruitment of AID to the appropriate DNA substrates (variable region genes for SHM and S regions for CSR) is not only essential for its function but also critical to protect the rest of the genome from AID-induced DNA damage. Indeed, mistargeting of AID activity to non-*Ig* genes has been implicated in chromosomal translocations and pathogenesis of B-cell lymphomas (Chiarle et al., 2011; Klein et al., 2011; Pasqualucci et al., 2008; Ramiro et al., 2004, 2006; Robbiani et al., 2008, 2009). AID expressed even at physiological levels in GC B cells or in *ex vivo* stimulated B cells associates with and deaminates a large number of non-*Ig* genes (Liu et al., 2008; Pavri et al., 2010; Yamane et al., 2011). It is generally believed that differential DNA repair protects non-*Ig* loci from sustained AID-induced damage, leaving only the *Ig* loci affected by AID activity (Liu et al., 2008). This hypothesis arose from the observation that AID activity could be detected outside the *Ig* loci on more than half of the transcribed genome (Liu et al., 2008). Nonetheless, while AID might appear to indiscriminately affect large parts of the genome, mistargeting of AID to non-*Ig* genes occurs at a much lower frequency. Indeed, the rate of mutation at the *Ig* loci is 20- to 100-fold greater than that found in any other loci (Liu et al., 2008), indicating the existence of an active *Ig* loci-specific AID-targeting mechanism in B cells.

8.1. Transcription-dependent AID recruitment

The observations that CSR, SHM, and mutations of non-*Ig* genes are tightly linked to transcription (Betz et al., 1994; Fukita, Jacobs, & Rajewsky, et al., 1998; Peters & Storb, 1996; Stavnezer-Nordgren & Sirlin, 1986; Winter et al., 1997; Yancopoulos et al., 1986) and that AID interacts with Pol II (Nambu et al., 2003; Pavri et al., 2010; Sun et al., 2013), suggested that transcription and Pol II-associated proteins might facilitate the binding of AID to target DNA sequences. In keeping with this notion, a short-hairpin RNA screen for effectors of CSR revealed that the Pol II-associated factor Spt5, which interacts with AID and colocalizes with paused Pol II, is required for CSR (Pavri et al., 2010). Depletion of Spt5 markedly reduces recruitment of AID to *Ig* and non-*Ig* sequences. Remarkably, occupancy of Spt5 on stalled Pol II sites is predictive of AID-dependent mutations at the

corresponding DNA sequence (Pavri et al., 2010). It is reasonable to conjecture that pausing of Pol II could be facilitated by R-loops impeding transcription elongation.

Histone modifications, like trimethyl histone H3 lysine 4 (H3K4me3) and hyperacetylated H3K9 (Ac-H3K9), that serve as markers of active chromatin conformation at promoter-proximal sites are observed throughout transcribed S regions (Wang, Whang, Wuerffel, & Kenter, 2006; Wang et al., 2009). A direct link between histone acetylation and CSR frequency was shown when inhibiting histone deacetylases through trichostatin A treatment led to increased Ac-H3K9 at S regions and CSR frequency (Wang et al., 2009). Additionally, deletion of PTIP, a component of several histone methyl transferase complexes, reduced H3K4me3 modifications at S regions and concomitantly impaired CSR (Daniel et al., 2010). Taken together, these data support a role for transcription-linked histone modifications in promoting the chromatin accessibility required for CSR.

8.2. GANP and 14-3-3 adaptors

The GC associated nuclear protein GANP (Kuwahara et al., 2004), which is induced in GC B cells and binds variable region transcripts has been reported to recruit AID to variable region genes during SHM. GANP deficiency impairs SHM without affecting CSR; however, the mechanism through which GANP mediates AID targeting to DNA during SHM is unclear (Kuwahara et al., 2004; Maeda et al., 2010).

The 14-3-3 adaptors represent a family of proteins with the ability to bind DNA with altered conformations (e.g., cruciform DNA) and have been implicated in numerous functions including DNA replication. In B cells undergoing CSR, expression of 14-3-3 proteins was rapidly induced through NF- κ B signaling and sustained by E2A (Mai et al., 2013). The 14-3-3 proteins bind to repeating RGYW motifs and transcribed S regions, and were shown to directly interact with AID, leading to the proposal that this family of scaffold proteins recruits AID to S regions. Surprisingly, the 14-3-3:AID interaction requires the C-terminus of AID (Xu et al., 2010), yet AID with C-terminal mutations is still able to target S μ (Barreto et al., 2003). Thus, the relevance of this interaction is not clear at present.

8.3. PTBP2: An AID interactor that promotes binding to S regions

Polypyrimidine-tract binding protein-2 (PTBP2), an RNA-binding protein originally thought to be expressed exclusively in the brain (thus referred to as brPTB or nPTB) and to regulate alternative mRNA splicing (Black, 2003), was subsequently detected in B cells in a proteomic screen to identify AIDinteracting proteins (Nowak, Matthews, Zheng, & Chaudhuri, 2011). Knock-down of PTBP2 in B cells significantly reduced the binding of AID to S regions and consequently impaired CSR. The mechanism by which PTBP2 targets AID to the *Igh* locus during CSR is not clear. PTBP2 could be recruited to S region DNA, possibly through interactions with other chromatin-associated factors to inhibit splicing, thus enhancing the stability of R-loops and facilitating CSR. Alternatively, the role of PTBP2 in CSR could be independent of its role in splicing. *In vitro* studies show that PTBP2 binds to sense and antisense S region RNA suggesting that PTBP2 may recruit AID to S regions through its interaction with S region transcripts (Nowak et al., 2011). Additional work is

required to elucidate the precise function of PTBP2 in CSR and its requirement, if any, in SHM.

In summary, several scenarios to explain the specific recruitment of AID to S regions have been proposed; yet the exact molecular mechanism(s) involved is still unclear. Our knowledge of the processes that determine AID targeting to variable region exons during SHM is even more limited, in part because there is no convenient assay or cell line to elucidate SHM. The use of the CH12F3 cell line and splenic B cells activated in culture have been valuable tools to identify not only AID but also other effectors of CSR; however, cell lines that undergo SHM (Ramos, BL2), which require weeks of growth in culture, have not similarly contributed to our understanding of SHM effectors. Additionally, the CH12F3 cell line and splenic B cells stimulated *ex vivo* do not undergo SHM, thus SHM has to be studied in the context of GC cells that are relatively difficult to acquire. Therefore, identification of SHM-specific AID-targeting factors awaits the identification of cell lines that undergo rapid, easily measurable SHM.

9. AID ACTIVITY BEYOND THE IG LOCI

While the variable region exons and S region DNA serve as natural targets of AID, it is becoming increasingly clear that AID can act at several non-*Ig* genes. “Off-target” AID activity could have pathological consequences such as in the development of B-cell lymphomas or could play a physiological role in the poorly understood process of epigenetic reprogramming.

9.1. AID in B-cell lymphomagenesis

GC B cells undergo robust proliferation and simultaneously undergo processes that induce mutations and DSBs, thereby predisposing these cells to oncogenesis (Kuppers, 2005). DNA-damage checkpoints and DNA repair proteins help to eliminate cells harboring unrepaired DSBs or oncogenic translocations (Franco et al., 2008). Yet, despite these safeguards, AID-induced aberrations do lead to oncogenesis. In fact, among human lymphomas it is estimated that about 95% are of B cell origin, and of these, a majority are classified as being derived from GC or post-GC B cells (Kuppers, 2005).

A hallmark of many B-cell lymphomas is chromosomal translocation between the *Ig* loci and a proto-oncogene, such as *Bcl2*, *Bcl6*, or *Myc* (Kuppers, 2005; Nussenzweig & Nussenzweig, 2010). These translocation events can place the oncogene under the control of the active *Ig* locus and result in constitutive oncogene expression. In this context, enhancers such as the 3'-RR can activate *c-Myc* over long distances and drive oncogene overexpression (Gostissa et al., 2009). In mouse models, AID was shown to be required for *c-Myc:Igh* translocations, and such translocations were readily observed in *ex vivo* stimulated splenic B cells (Ramiro et al., 2004, 2006). Significantly, while S regions serve as physiological substrates for AID, an elegant study demonstrated that DSBs at *c-Myc* were also AID dependent (Robbiani et al., 2008). Additionally, studies mapping DSBs or highthroughput genome-wide translocation sequencing demonstrated that AID generates off-target DSBs at many genes in activated B cells, including many genes associated with B-cell oncogenesis (Chiarle et al., 2011; Klein et al., 2011; Staszewski et al., 2011). Interestingly, many

translocations were also observed in AID-deficient cells, indicating that certain genomic regions may be prone to DSB formation (Chiarle et al., 2011; Klein et al., 2011). This is consistent with an earlier observation that a small fraction of *c-Myc:Igh* translocations can also occur in the absence of AID (Unniraman, Zhou, & Schatz, et al., 2004). Both AID-dependent and - independent DSBs are associated with transcription, which could generate AID targets through collision with replication forks or through the formation of R-loops. It has also been proposed that collaboration between RAG1/2 and AID activities could promote chromosomal translocations. AID was shown to be expressed in developing B cells in the bone marrow (Han et al., 2007), and it has been proposed that AID deamination at certain CpG sites generates noncanonical RAG1/2 and Artemis substrates, leading to DSBs. The concerted activities of RAG1/2 and AID could potentially explain translocation hotspots in human cancers originating from developing B cells (Cui et al., 2013; Tsai et al., 2008).

It is important to note that not all translocations lead to B-cell lymphomas. This is best exemplified from AID overexpression studies using transgenic mouse models to assess the oncogenic ability of dysregulated AID expression. Mice with constitutive and ubiquitous expression of AID succumb to T-cell lymphomas along with developing lung adenocarcinoma (Okazaki et al., 2003). Strikingly, B cell tumors were not observed, either due to a longer latency of these tumors or due to a protective mechanism present in B cells. The idea that B cells may be protected from aberrant AID expression was given weight when a B cell-specific transgenic mouse line, driven by the CD19 promoter, was found to have reduced CSR and SHM, indicating that the transgenic AID expressed was being inactivated (Muto et al., 2006). In agreement with this finding, transgenic mice with AID expression controlled by the *Igk* regulatory elements developed lymphomas derived from B cells over a broad spectrum of stages only in the context of p53 deficiency (Robbiani et al., 2009). Interestingly, even though the *Igk* transgenic mice in a p53-proficient background did not generate B-cell lymphomas, B cells isolated from these animals possessed widespread genomic instability, including mutations and translocations at regions beyond the *Ig* loci (Robbiani et al., 2009).

Finally, spatial organization of the mouse genome might have a role in directing chromosomal translocations. A combination of high-resolution (Hi-C) depiction of the spatial organization of the mouse pro-B cell genome and high-throughput genome-wide translocation mapping of RAGinstigated DSBs showed that translocations were highly enriched in *cis* along a single chromosome, and that interchromosomal translocations occur in a manner directly related to pre-existing spatial proximity (Zhang et al., 2012). However, it is not entirely clear if AID-instigated interchromosomal translocations are determined by proximity between the *Igh* locus and translocation partners (Rocha et al., 2012), or by the extent and persistence of AID-induced damage (Hakim et al., 2012), or a combination of both scenarios. For a more comprehensive discussion of genomic integrity in cells undergoing antibody diversification, please read a recent review by Alt and colleagues (Alt et al., 2013).

Translocations are not the only transforming events arising from AID activity. SHM-like activity has also been implicated in contributing to transforming events that give rise to lymphomagenesis. A study assessing mutations within proto-oncogenes in diffuse large-cell

lymphomas revealed that ~50% of the tumors analyzed contained mutations in *Pim1*, *c-Myc*, *RhoH*, or *Pax5* genes and a mutation profile reminiscent of SHM (Pasqualucci et al., 2001). Mutations in the *Bcl6* gene, whose expression is imperative for proper GC function, were also found within a region that was previously linked to chromosomal translocations in lymphoma cells and in B cells isolated from human tonsillar GCs (Migliazza et al., 1995; Pasqualucci et al., 1998). Similarly, the *CD95/Fas* gene, which is also actively expressed in GCs, was found to acquire somatic mutations during the GC reaction (Muschen et al., 2000). It is important to highlight that some of the mutations described above could be the result of increased mutagenicity upon transformation of the GC B cell and/or a global defect in DNA repair pathways. However, this does not seem to be the case with *Bcl6* and *CD95/Fas*, as these genes appear to be directly targeted for mutation during the normal GC reaction (Muschen et al., 2000; Pasqualucci et al., 1998).

9.2. AID activity beyond B cells: Epigenetic reprogramming

While AID activity in SHM and CSR has been well studied, the observation that AID can deaminate methylated cytidines (Morgan, Dean, Coker, Reik, & Petersen-Mahrt, 2004) raised the possibility that AID could have roles beyond antibody diversification. It was discovered that AID is expressed in certain non-B cell populations, particularly in primordial germ cells (PGC), embryonic stem cells, oocytes, and somatic cells undergoing reprogramming to the pluripotent state (Bhutani et al., 2010; Kumar et al., 2013; Morgan et al., 2004). It was therefore proposed that AID mediates DNA demethylation through an indirect mechanism that involves deamination of 5'-methylated cytidines (5mC) with the subsequent replacement of the resulting thymidine with an unmethylated cytidine through the concerted activities of the BER pathway (Fig. 1.9).

A rapidly accumulating body of work provided strong evidence that active DNA demethylation is quite prevalent in mammals. Following fertilization in mammals, the paternal pronuclei undergo extensive loss of 5mC in a process that is both rapid and independent of cell division, thus providing a classic example of active demethylation (Oswald et al., 2000). Genomewide epigenetic reprogramming also occurs in the PGCs after they have reached the embryonic gonads at embryonic days E10.5–13.5 (Popp et al., 2010). In this case, demethylation is truly global with genome-wide erasure of DNA demethylation marks at most promoters and genic, intergenic, and transposon sequences being hypomethylated at this stage (Popp et al., 2010). Active DNA demethylation has also been proposed as a gene-regulation mechanism in selected adult tissues, the most striking evidence being demethylation at the promoter of brain-derived neurotrophic factor and fibroblast growth factor 1 in postmitotic (and thus nonreplicating) neurons in animal models of emotional stress (Martinowich et al., 2003). Recent studies have implicated a role of AID and the *ten-eleven* translocation (TET) family of proteins in this active demethylation reaction (Guo, Su, Zhong, Ming, & Song, 2011). Finally, active demethylation appears to be a critical component of experimental reprogramming to a pluripotent stem cell state (Bhutani et al., 2010; Kumar et al., 2013).

Initial support of the requirement of AID in active demethylation came from genome-wide bisulphite-sequencing studies, which demonstrated a significant increase in global

methylation in AID-deficient PGCs relative to wild-type cells (Popp et al., 2010). Additionally, in a heterokaryon-based reprogramming assay, AID was found to interact with the promoters of the pluripotency genes *Oct4* and *Nanog*, which are rapidly demethylated during reprogramming (Bhutani et al., 2010). Short-hairpin-induced knock-down of AID led to a significant decrease in the demethylation at these promoters (Bhutani et al., 2010). In a compelling recent study, AID-deficient fibroblasts transduced to express the four pluripotency factors Oct4, Klf4, Sox2, and c-Myc were found to be defective in their ability to be stably reprogrammed into induced pluripotent stem (iPS) cells (Kumar et al., 2013). While the AID-deficient cells initiated the reprogramming process and expressed early markers of iPS cells, after 3 weeks in culture they were found to be significantly hypermethylated compared with wild-type cells and failed to express genes involved in the secondary pluripotency network, thereby leading to redifferentiation (Kumar et al., 2013).

While the above data seem to suggest a role for AID in demethylation, this activity might be limited to defined settings, such as during development and reprogramming of specific cells, for example, fibroblasts. AID is expressed most abundantly in stimulated B cells; however, reduced bisulphite-sequencing analysis from wild-type, AID-deficient, and AID overexpressing B cells revealed no differences in methylation status between the AID genotypes (Fritz et al., 2013). This highlighted the possibility that B cells can subvert the demethylase activity of AID as a protective mechanism during secondary antibody diversification reactions. It is also important to note that any role AID may have in demethylation is not absolutely required during development, as AID-deficient mice are viable, despite having smaller litter sizes (Kumar et al., 2013; Muramatsu et al., 2000; Popp et al., 2010).

A possible alternative pathway for AID-mediated demethylation involves a two-step process whereby the 5mC is first oxidized to hydroxymethylcytosine (5hmC) by the TET proteins and subsequently converted to 5-hydroxymethyluridine (5hmU), which can be repaired by thymine DNA glycosylase (TDG) (Cortellino et al., 2011). In support of this idea, TET1 overexpression increased demethylation through the formation of 5hmC and subsequent BER, while overexpression of AID led to a decrease in 5hmC levels (Guo et al., 2011). Expression of AID in the mouse brain also significantly reduced 5hmC levels. While endogenous AID expression in the brain was not detectable, APOBEC1 was expressed in these neuronal populations and was similarly able to decrease 5hmC levels (Guo et al., 2011). Although these studies suggest a role for AID in this process, the activity of AID on 5hmC has not been demonstrated in a physiologically relevant setting. Furthermore, biochemical studies coupled with measurements of steric constraints using purified proteins have argued against AID or APOBEC1 acting on 5hmC residues (Nabel et al., 2012). Thus, the potential for AID to serve as a demethylase in this capacity, and as a demethylase in general, is an exciting new area of research.

10. PERSPECTIVES

The discovery of AID was one of the most significant advances in our understanding of the mechanism of CSR and SHM. Work over the past decade has provided us with a model that mechanistically links germline transcription and S region sequence to AID-

mediated DNA deamination. We now have an understanding of how the BER, MMR, and C-NHEJ/A-EJ DNA repair pathways participate in the recombination reaction. There have also been significant advances made in our understanding of how potent a mutator AID is and how multiple processes come together to regulate the activity of this enzyme. Finally, we are beginning to unmask potential roles of AID beyond the immune system, such as in the maintenance of the pluripotent stem cell state. Yet, despite these advances, several key issues remain unresolved.

Several AID-interacting proteins that have the potential to recruit AID specifically to the relevant regions of the *Ig* loci have been identified, but there is still no coherent model that clearly explains how AID activity under physiological conditions is so exclusively specified for the *Ig* loci. Additionally, we know that multiple DNA repair pathways participate in CSR; however, these pathways are ubiquitous and have evolved to prevent the incorporation of mutations and deletions of genomic sequences. Thus, it is curious how the immune system manages to subvert normal repair machineries and instead hijacks components of these pathways to promote mutations and the persistence and synapsis of DSBs that are over 100 kb apart. The molecular basis underlying the balance between normal and aberrant repair requires further elucidation. Finally, with regard to the potential role of AID as a DNA demethylase, it remains to be resolved how this demethylase activity can be reconciled with the mutagenic abilities of AID. A combination of biochemical and novel *in vivo* genetic manipulations will be required to address these exciting issues, which will be at the forefront of AID research for the next several years.

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Figure 1.1.

Secondary immunoglobulin gene diversification. Mature B cells undergo class-switch recombination (CSR) and somatic hypermutation (SHM). During SHM, mutations are introduced into the rearranged variable region genes. CSR is initiated by transcription (grey arrows) through switch (S) regions (ovals) and requires AID and components of several DNA repair pathways. During CSR, the intervening DNA sequence between participating S regions is excised as a switch circle and a new constant region gene is juxtaposed downstream of the variable region exons.

Figure 1.2.

Model for SHM. AID deaminates dCs to dUs within RGYW hotspots in variable region genes. Activities of mismatch repair (MMR) and base excision repair (BER) proteins (e.g., UNG of the BER pathway) coupled with DNA replication generate transition and transversion mutations.

Figure 1.3.

Germline transcription through S regions. Each constant region gene is comprised of a transcription unit with a cytokine-inducible promoter (P), an intervening (I)-exon, S region, and *C_h* exons. The primary transcript is spliced and polyadenylated to generate a noncoding mature transcript.

Figure 1.4.

Transcription-driven R-loop formation at S regions. Transcription occurs through each participating S region (indicated by arrows) and the nascent transcripts (dotted lines) remain bound to template DNA due to high GC content of the sequence. This displaces the non-template strands as ssDNA substrates, forming structures called R-loops.

Figure 1.5. Processing of deaminated DNA during CSR. S regions deaminated by AID are processed by UNG, APE1, and MMR proteins to generate blunt DSBs that are synapsed and ligated to complete CSR.

Figure 1.6.

Phosphorylation and DSB-dependent positive feedback loop amplifies DSB formation. Multiple AID molecules are assembled at S region DNA. An initial DNA break due to AID-mediated DNA deamination of cytidines is generated and can result in inefficient CSR. ATM-dependent S38 phosphorylation of AID induces the recruitment of APE1, which increases the number of DNA breaks generated. This induces additional phosphorylation of AID at S38 and APE1 recruitment to generate the high density of DSBs required for efficient CSR. AID phosphorylation at S38 also allows for interaction with RPA, which promotes efficient CSR potentially by recruiting downstream repair factors.

Figure 1.7. Regulation of AID. AID expression, localization and activity are regulated through multiple processes.

Figure 1.8.

Transcription factor-binding sites in *Aicda* locus. The *Aicda* locus is regulated by four regions of transcription factor-binding sites. Region 1 is just 5' of the transcription start site (TSS), region 2 is located between exons 1 and 2 (shown in black), region 3 is ~17 kb downstream of the TSS, and region 4 is ~8 kb upstream of the TSS. *Adapted from* Lee-Theilen and Chaudhuri (2010).

Figure 1.9.

AID as an indirect demethylase. The deaminase activity of AID can lead to demethylation through two potential pathways. In one, AID can deaminate methylated cytidines (5mC) to generate thymidine. In the other, AID can deaminate hydroxymethylcytosine (5hmC) that is generated by the oxidation of 5mC by the ten-eleven translocation (TET) proteins. In both pathways, the mismatch is repaired by thymine DNA glycosylase (TDG) and BER components. The end-product is the replacement of methylated cytidines with unmethylated cytidines.