Regulation of immunoglobulin diversification pathways by AID in a transgenic mouse model

A thesis

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ABSTRACT

The activation-induced cytidine deaminase (AID) enzyme plays a central role in B-cell diversification such as somatic hypermutation (SHM), immunoglobulin (Ig) V-gene conversion, and class switch recombination (CSR). AID function has also been implicated in chromosomal B-cell translocations between the c-myc oncogene and the immunoglobulin heavy-chain (Igh) locus. The regulatory mechanisms that promote such vastly different outcomes after AID deamination are not clear.

To better understand AID-dependent pathways, I have used a transgenic mouse, which carries an antibody (Ab) heavy chain transgene, to monitor CSR, SHM, and Ig V-gene conversion. I have shown that Igh/transgene translocations require AID and resemble interchromosomal CSR events, although the recombination mechanism does not appear to involve trans-switching between the transgene Sμ region and the endogenous Sμ region. Because trans-switching between the same S regions would not generate a change in Ab class, these results suggest that CSR may be regulated to maximize B-cell responses to antigens.

My results also show that, although Ig V-gene conversion is not detected at the transgene locus, the transgene is capable of accumulating AID-induced mutations, which are dependent on the function of the Base Excision Repair (BER) and Mismatch Repair (MMR) pathways. These results indicate that Igh *cis*

elements 5' of the Cμ gene are sufficient to promote the mutagenic function of BER and MMR, and that missing *cis* elements downstream of the Cμ gene probably function to recruit AID to Ig loci more efficiently, which in turn may be needed for gene conversion. Furthermore, because double stranded DNA breaks (DSBs) may be needed for Ig V-gene conversion, these results imply that the extent of DNA damage may dictate how the damage is resolved. Finally, I show that p21 is dispensable for SHM and CSR.

Together, my results indicate that one of the functions of the Ig regulatory elements may be to make AID deamination more efficient, which in turn, may determine how AID-induced DNA damage is resolved.

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CHAPTER 1

INTRODUCTION

Throughout the course of evolution, the immune system has developed to protect multicellular organisms against a vast variety of different pathogens. As part of the specific (adaptive) immune response, B-cells have evolved to produce immunoglobulin (Ig) receptors, which recognize and bind to a specific region (epitope) of a given pathogen, known as the antigen (Ag) region or antigenic determinant. The specificity is achieved by the generation of nearly infinite repertoire of B-cells, each with an unique Ig receptor, through the process of Ig gene rearrangement known as V(D)J recombination (1). Additional mechanisms such as Ig V-gene conversion, somatic hypermutation (SHM), and class switch recombination (CSR) add further to Ig gene diversity, however, there are variations among the usage of these Ig diversification mechanisms among different species (2-4). Additionally, all of the mechanisms that are involved in generating B-cell diversity rely on extensive DNA editing that can be detrimental to cells if not regulated properly, causing aberrant chromosomal recombinations which are hallmarks of many types of lymphomas (5). One enzyme, activation-induced cytidine deaminase (AID), is responsible for the initiation of Ig diversifications, such as SHM, Ig V-gene conversion, and CSR, and if not regulated properly, potentially can also initiate aberrant chromosomal recombination (6-10). Among the most interesting questions in the field are deciphering how AID is able to initiate such diverse pathways and what are the regulatory mechanisms that protect the genome from indiscriminate AID activity.

1. V(D)J recombination produces the primary B-cell repertoire with unique immunoglobulin receptors.

1.1 The structure of an immunoglobulin molecule

The Ig receptor is expressed on the surface of B-cells and referred to as the B-cell receptor (BCR). The Ig, or antibody (Ab), is the secreted form of the BCR. The Ig molecule is composed of two types of protein chains, the heavy chain (H-chain) and the light chain (L-chain). Two identical H-chains and two identical L-chains, joined by disulfide bonds, make up each Ig molecule. The variable domains (V-region) of the heavy and light chains together makes up the Ab binding site, or the region where the Ab binds a specific Ag. The V-region amino acid sequences that are specific for a given Ag have high amino acid sequence variability as compared to the rest of the V-region. These hypervariable regions are called complementarity-determining regions (CDRs) and there are three CDR regions for each of H-chain and L-chain, namely CDR1, CDR2, and CDR3 (Figure 1). The constant domains of the heavy and light chains make up the constant region (C-region) (1). The C-region of the H-chain determines how the Ag is removed from the body (1). The H-chain and the L-chain are encoded on different chromosomes. There is one type of H-chain, located on mouse chromosome 12, and two types of L-chain, lambda (λ) and kappa (κ), located on mouse chromosome 16 and 6 respectively (11). A given Ab either has the λ or κ chain.

1.2 V(D)J recombination

1.2a Combinatorial diversity. B-cells acquire diverse Ig receptors during their development in the bone marrow (BM) (12). The recombination of variable (V), diversity (D), and joining (J) Ig gene segments produces the V-region of the Ab. Because species like humans and mice have many V, D, and J gene segments, the joining of different combinations of V, D, or J gene segments, termed combinatorial diversity, produces an immense amount of different Ab molecules (Figure 1, 2). Additionally, junctional diversity which is the introduction and subtraction of nucleotides at the junctions, caused by the recombination process, adds further to the diversity of Ab molecules (1).

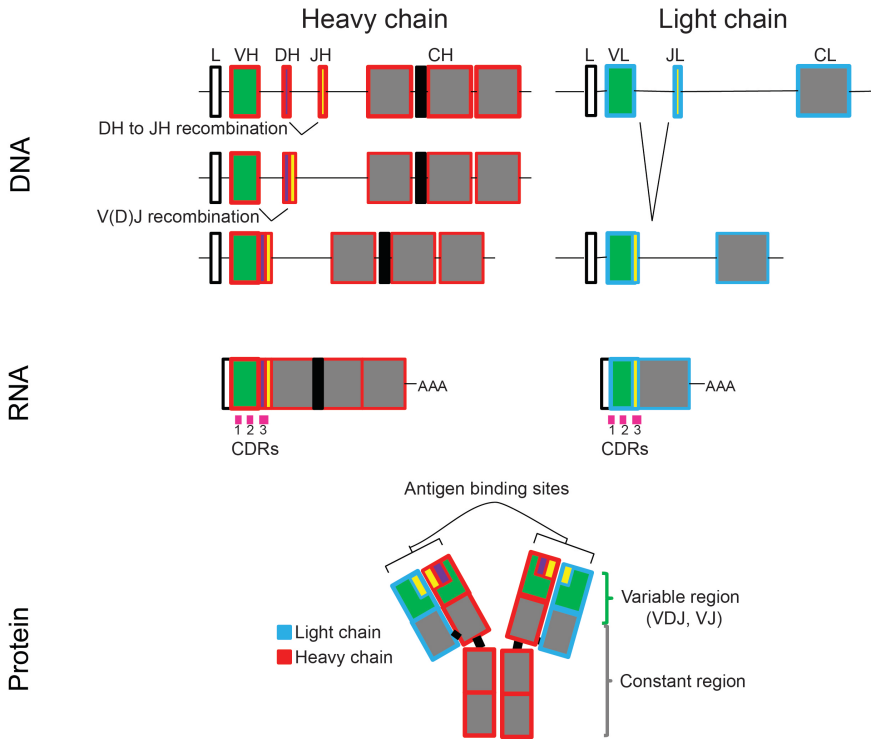

Figure 1

Figure 1. The antibody structure. Antibodies are composed of two types of protein chains, the heavy chain (red) and the light chain (blue), joined by disulfide bonds (black). V(D)J recombination at the DNA level make up the variable regions of the antibody. The V-regions of both the light chain and the heavy chain make up the antigen binding sites. The amino acid sequences that are specific for a given antigen have high amino acid sequence variability as compared to the rest of the V-region and called complementarity-determining regions (CDRs). There are three CDR regions for each of heavy chain and light chain, namely CDR1, CDR2, and CDR3 (pink lines). CDR1 and CDR2 are in the V region, while CDR3 spans the V(D)J junctions. The constant genes of the heavy and light chains make up the constant region. Diagram not drawn to scale. L: leader exon.

1.2b The IgH and IgL loci. The mouse Ig H-chain (Igh) locus spans about 2,300 kilobases (kb), carries about 152 V_H genes comprising 15 families, 17-20 D_H genes (depending on the mouse strain), 4 J_H genes, and 8-9 C_H genes (depending on the mouse strain) (11). The classification of an Ig V-gene family is based on the nucleotide sequence identity where family members are at least 80% identical (13). The mouse Ig κ L-chain ($Ig_κ$) spans about 3,200 kb, carries about 174 Vκ genes comprising about 19 families, 5 Jκ genes, and one Cκ gene. The mouse Igλ L-chain spans about 240 kb, carries 3 Vλ genes (8 Vλ in wild mouse strains) comprising 2 families, 5 Jλ genes, and 4 Cλ genes (11). It should be noted, however, that not all of the V(D)J genes have the appropriate Ig *cis* elements that are needed for the recombination machinery, and therefore, are not functional and are referred to as pseudo (Ψ) genes (14).

1.2c Allelic exclusion. V(D)J recombination occurs in a cell-stage specific manner and ensures that each individual B-cell expresses one unique Ig receptor despite the fact that two alleles of every Ig receptor are available for recombination (Figure 2). This is achieved by step-wise V(D)J recombination and allelic exclusion of the non-functional alleles (15, 16). For example, D_H to J_H rearrangement occurs first on one Igh allele, followed by V_H to D_HJ_H rearrangement to produce a functional V-region. Expression of a functional Ig receptor inhibits further V(D)J recombination on the second allele, referred to as allelic exclusion, ensuring that only one unique Ig receptor is expressed. It should be noted, however, that because of junctional diversity, there is a high

probability that the introduction and subtraction of nucleotides at the V(D)J recombination junctions may disrupt the triplet codon reading frame and splice junctions, and therefore not produce a full length protein. Only a small fraction, one third, of V(D)J rearrangements produce functional full length protein, termed productive V(D)J rearrangement (1). The V(D)J recombination process is regulated such that if rearrangement on one chromosome produces a non-productive (non-functional) variable region, then rearrangement continues on the second chromosome (15, 16). Chromosomal modifications associated with transcriptionally active chromatin regulates the choice between recombination of the different V(D)J genes (15).

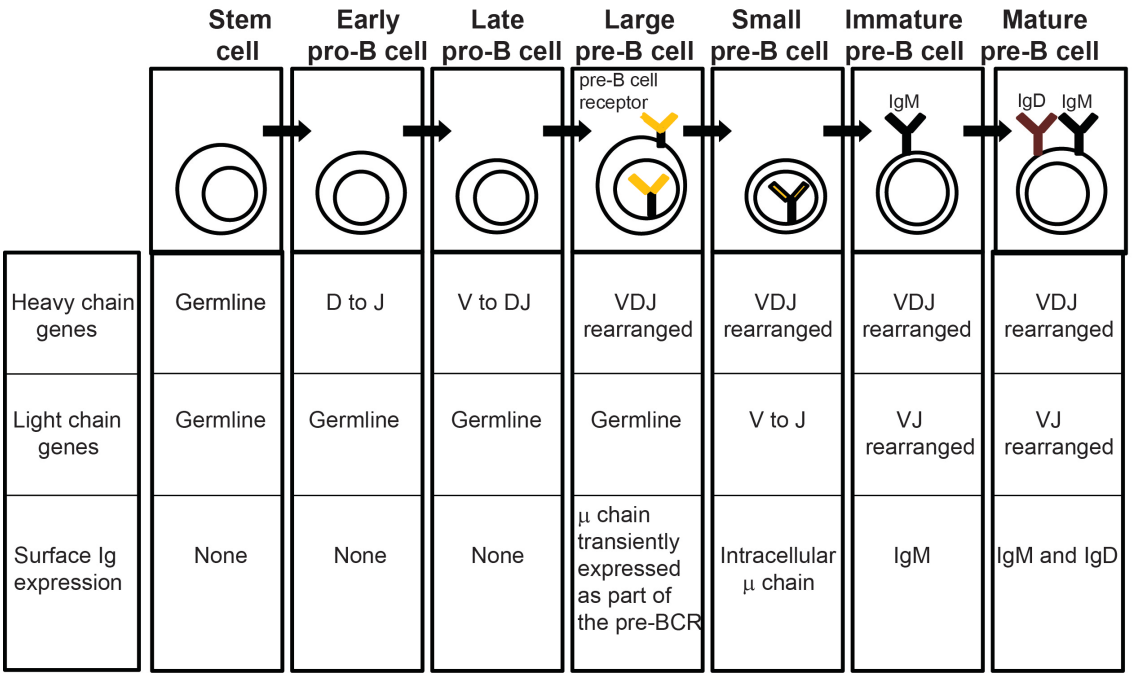

Figure adapted from Janeway CA, Immunobiology, 5th edition, *Garland Sciences*, New York 2001

Figure 2

Figure 2. B-cell development. Immunoglobulin diversification starts in the bone marrow where D_H to J_H recombination, on one heavy (H) chain allele, occurs first at the early pro-B cell stage, followed by V_H to D_HJ_H recombination at the pro-B cell stage. Light (L) chain locus is still in germline configuration. Successful VDJ recombination creates a functional V region at the H chain and leads to the expression of the pre-B-cell receptor which is composed of μ chain with a surrogate L-chain. If VDJ recombination does not generate a functional V region, recombination continues on the second H-chain allele. At the small pre-B cell stage, L-chain VJ recombination starts. IgM is expressed on the surface of the B-cell after successful V(D)J recombination. At the mature B-cell stage, both IgM and IgD are expressed on the surface of the B-cell. IgD is made via alternative splicing of transcripts that contain both IgM and IgD.

1.2d Rag enzymes. The molecular mechanism of V(D)J recombination involves binding of the enzymes recombination activation gene (Rag)-1 and Rag-2 to recombination signal sequences (RSS) located adjacent to each V, D, and J gene segments (17). The Rag proteins bind the pair of RSS to be joined together and then cleave the DNA causing DNA double-stranded breaks (DSBs). This creates hairpin structures at the ends of the Ig gene segments which are resolved by binding of DNA repair proteins that are part of the non-homologous end joining (NHEJ) pathway (17).

2. Activation-induced cytidine deaminase is required for additional immunglobulin gene diversity by initiating immunoglobulin V-gene conversion, somatic hypermuation, and class switch recombination.

2.1 Activation-induced cytidine deaminase function

Activation-induced cytidine deaminase (AID, gene name is *Aicda*) was identified in 1999 by a cDNA subtraction method in a B-cell line that was activated for CSR (6). The role of AID in Ig diversification was confirmed by deleting the gene in mice and in chicken cell lines to show that SHM, CSR, and Ig V-gene conversion were abolished (7-9). Furthermore, humans with hyper-IgM syndrome (HIGM-2) were shown to have mutations in the AID gene and were thus unable to undergo SHM and CSR (18, 19). Initially it was suggested that AID was a RNA editing enzyme because AID has high amino acid sequence homology to the mRNA

editing enzyme APOBEC-1 (apolipoprotein (apo) B mRNA editing catalytic polypeptide 1) (6). Because APOBEC-1 deaminates cytosine (C) to uracil (U) at a specific position of apoB100 mRNA, generating a mRNA (apoB48) that encodes a protein with a different biological function (20), it was proposed that AID may function similarly, deaminating a precursor mRNA that encodes a yet to be discovered endonuclease that is involved in Ig diversification (6). The RNA editing hypothesis was further supported by the findings that *de novo* protein synthesis was required downstream of AID expression, as levels of CSR was decreased in the presence of protein synthesis inhibitors (21). Subsequent studies, however, have shown that AID deaminates cytosine residues to uracils on single stranded DNA (ssDNA) generating U:G mismatches (22-25). In addition, *in vitro* studies have further shown that AID does not deaminate double-stranded DNA (dsDNA), DNA/RNA hybrids, or RNA in any form (26-29). The detection of uracil residues in Ig V-regions and Ig switch regions have provided compelling evidence that AID functions by deaminating cytosine residues on DNA (30). The cytosine deamination activity of AID leads to an U:G mismatch, and depending on how the U is processed, will generate mutations and DNA breaks needed for Ig V-gene conversion, SHM, and CSR which will be discussed below.

2.2 Immunoglobulin V-gene conversion

2.2a Homologous recombination. Homologous recombination (HR) is a DNA repair pathway that utilizes homologous DNA sequences to repair DNA breaks.

The molecular mechanism of HR is straightforward: 1) the 5' ends of DSBs are processed to yield 3' ssDNA tails; 2) the 3' ssDNA tails will actively "scan" the genome for homologous sequences and invade the homologous DNA duplex forming a displacement (D)-loop which is extended by DNA synthesis; and 3) the extended strand is dissociated and annealed back with the original strand (Figure 3, (31)). Gene conversion is a special type of HR that involves non-reciprocal transfer of genetic material from a donor DNA segments to recipient DNA segments. The donor DNA segment remains unaltered while part, or all, of the recipient DNA segment becomes identical to that of the donor DNA segment (32). There are several pathways that will generate a gene conversion event, i.e. non-reciprocal transfer of DNA segments. One example is called synthesis dependent strand-annealing (SDSA) where after D-loop extension, the newly extended invading strand is displaced and annealed to the other 3' end of the double-stranded break, leading to the formation of only gene conversion because crossover events, i.e. reciprocal exchanges of DNA segments between recombining chromosomes, does not occur (32). Alternatively, the extended D-loop can join with the other 3' ssDNA tail, which is referred to as second-end capture because the other 3' end of the double-stranded break is captured in the D-loop, and DNA synthesis and ligation of the DNA results in the formation of double Holliday junctions, i.e. an intermediate at which the strands of two dsDNA molecules cross. Depending on how the Holliday junctions are resolved, a crossover (reciprocal exchange) or non-crossover (non-reciprocal exchange) of DNA segments can occur (Figure 3, (32)). The DNA segments involved can be

alleles on homologous chromosomes, tandem copies of a gene on the same chromosome, tandem genes interacting from sister chromatids, or a pair of related genes located anywhere in the chromosome (32). Gene conversion can occur during mitosis and meiosis (32), and it is often associated with the DNA synthesis (S) phase of the cell cycle (32).

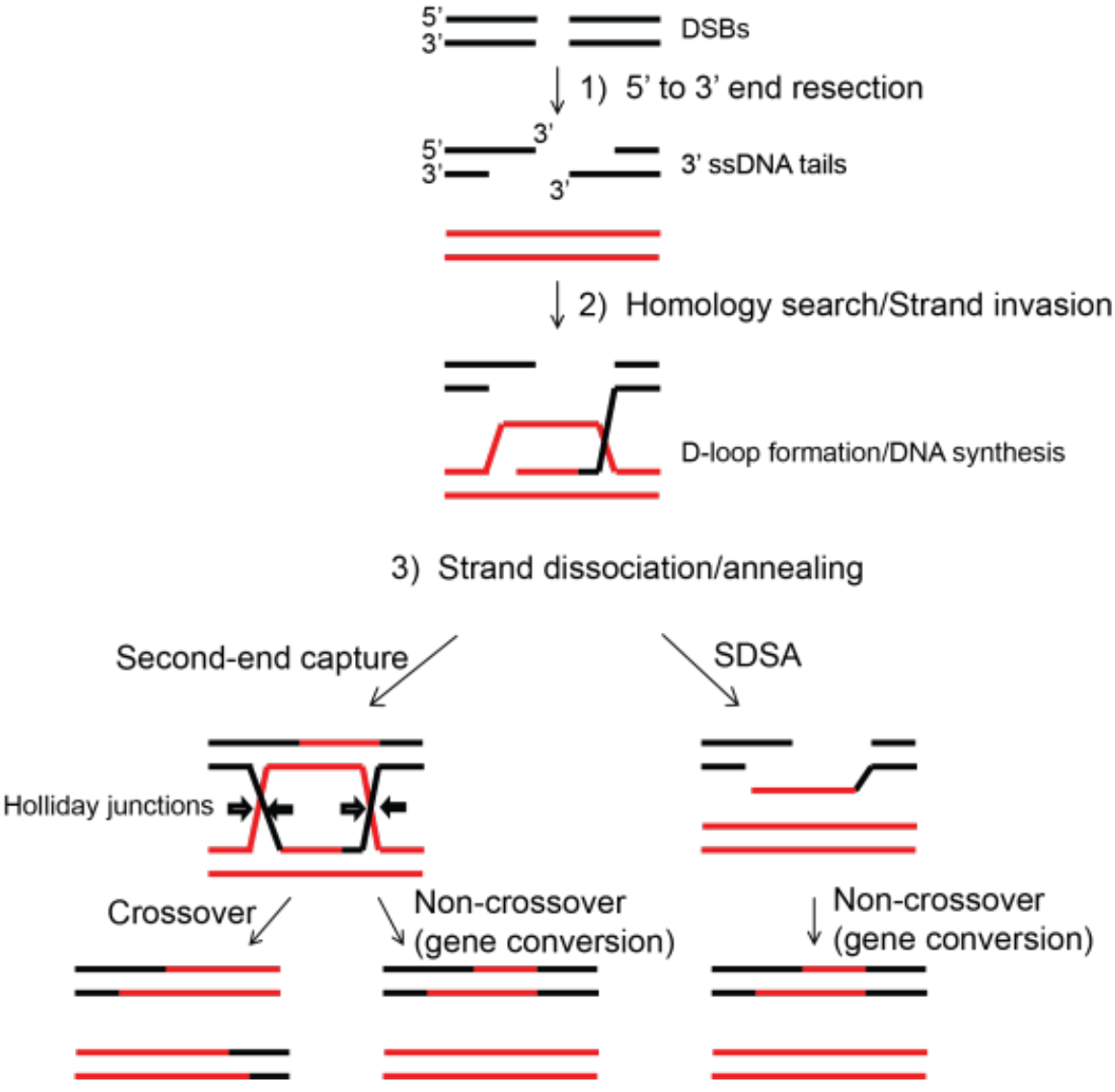

Figure 3

Figure 3. Homologous recombination. Double-stranded breaks (DSBs) are needed for homologous recombination (HR) which are resected to generate 3'single-stranded DNA (ssDNA) tails. The 3'ssDNA scan the genome for homologous sequences and invade the homologous DNA strands (red) forming a displacement (D) loop. DNA synthesis follows using the homologous DNA strand as a template. The newly synthesized DNA can dissociate from the D-loop and anneal back to the original DNA strand in what is called synthesis dependent strand annealing (SDSA) which generates a non-reciprocal transfer of DNA segments from the donor (red) to the recipient (black) regions (non-crossover or gene conversion). Alternatively, the extended D-loop can join with the other ssDNA break, which is referred to as second-end capture, and DNA synthesis and ligation results in the formation of an intermediate at which the strands of two double-stranded DNA (dsDNA) molecules cross called Holliday junctions. Depending on how the Holliday junctions are resolved (arrows), a crossover (reciprocal exchange) or non-crossover (non-reciprocal exchange) of DNA segments can occur.

2.2b Proteins involved in homologous recombination. Much of what is known about HR has been from studies in yeast and many of the proteins involved are conserved in higher eukaryotes. Essentially, the 5' ends of the DSBs are processed by 5'→3' nucleases to form the 3' ssDNA tails (overhangs). This function is performed by the MRN complex (Mre11-Rad50-Nbs1), Exo1, Dna2, and Sae2 (human CtIP) proteins (32). It is reported that Mre11 removes about 50-100 nucleotides while additional resection is achieved by Exo1 (32). The 5' end can be processed for long distances, up to 1 kb (32). The naked 3' ssDNA overhangs are then coated with the ssDNA binding protein RPA, which prevents the ssDNA from forming secondary structures (32). Scanning for, and invasion of, homologous DNA sequences by the 3' ssDNA tail is dependent on Rad51, which is similar to *Escherichia coli* (*E.coli*) RecA protein (33).

Since RPA has strong affinity for ssDNA, displacing RPA and loading Rad51 onto the 3' ssDNA tail requires additional proteins which are referred to as mediator proteins (32). Among these mediator proteins are the Rad51 paralogues: Xrcc2, Xrcc3, Rad51B, Rad51C, Rad51D; and the homologous recombination protein Rad52 (33). The Rad51 paralogues have DNA binding activity and can facilitate Rad51 loading onto the DNA (32). The function of Rad52 is also to assist loading of Rad51 to the 3' ssDNA tails (33), although Rad52 has an additional role in DNA repair that is independent of Rad51, involving the annealing of two 3' ssDNA tails, a process termed single-stranded annealing (SSA) (33). Finally, another group of mediator proteins, not found in

yeast, that assist in loading of Rad51 onto the 3' ssDNA tails are the breast cancer susceptibility proteins (BRCA1 and BRCA2) (33).

Once Rad51 is loaded onto the 3' ssDNA tails, homology search and strand invasion begin. Strand invasion is further aided by Rad54 which is a dsDNA ATPase, similar to the Swi/Snf family of chromatin remodeling factors capable of sliding nucleosomes and stimulating DNA strand invasion by Rad51 (33). A more important function of Rad54 may be the ability to displace Rad51, promoting the transition from strand invasion to D-loop formation and DNA synthesis (33). The final step in the homologous recombination pathway is the dissolution of the Holliday junctions which are carried out by BLM in humans and Sgs1 in yeast (33).

2.2c Immunoglobulin V-gene conversion. The diversification of the B-cell repertoire in some species like birds can occur by gene conversion (2, 34). This process is referred to as Ig V-gene conversion throughout this paper and it has been well characterized in chickens (35, 36). The chicken H-chain contains only one functional V_H gene, one functional J_H gene, and about 15 functional D_H genes that are highly homologous to each other, in some cases 100% homologous (37). VDJ rearrangement, therefore, generates very limited B-cell repertoire, mostly due to some junctional diversity. Similarly, the L-chain contains one functional V_L gene, one functional J_L gene, and because chickens only have one L-chain, the primary B-cell repertoire will have one functional VJ

region (37). Upstream of the functional V(D)J regions, at both the L-chain and H-chain loci, are many pseudo (Ψ) V genes (~80 ΨV_H and ~25 ΨV_L) that lack RSSs, promoters, or are truncated, i.e. non-functional and unable to participate in V(D)J recombination, but do participate in Ig V-gene conversion by acting as the donor sequences (37). The B-cell repertoire is expanded by the non-reciprocal transfer of DNA sequences from the upstream ΨV genes to the downstream functional V(D)J region (Figure 4, (35, 36)). The gene conversion tracks, or segments of DNA sequences that are introduced into the functional V(D)J segments, are about 10-300 bp long, most of the differences being at 3' region (37). The ΨV genes are fairly homologous to each other, where most of the differences cluster around the CDRs (37).

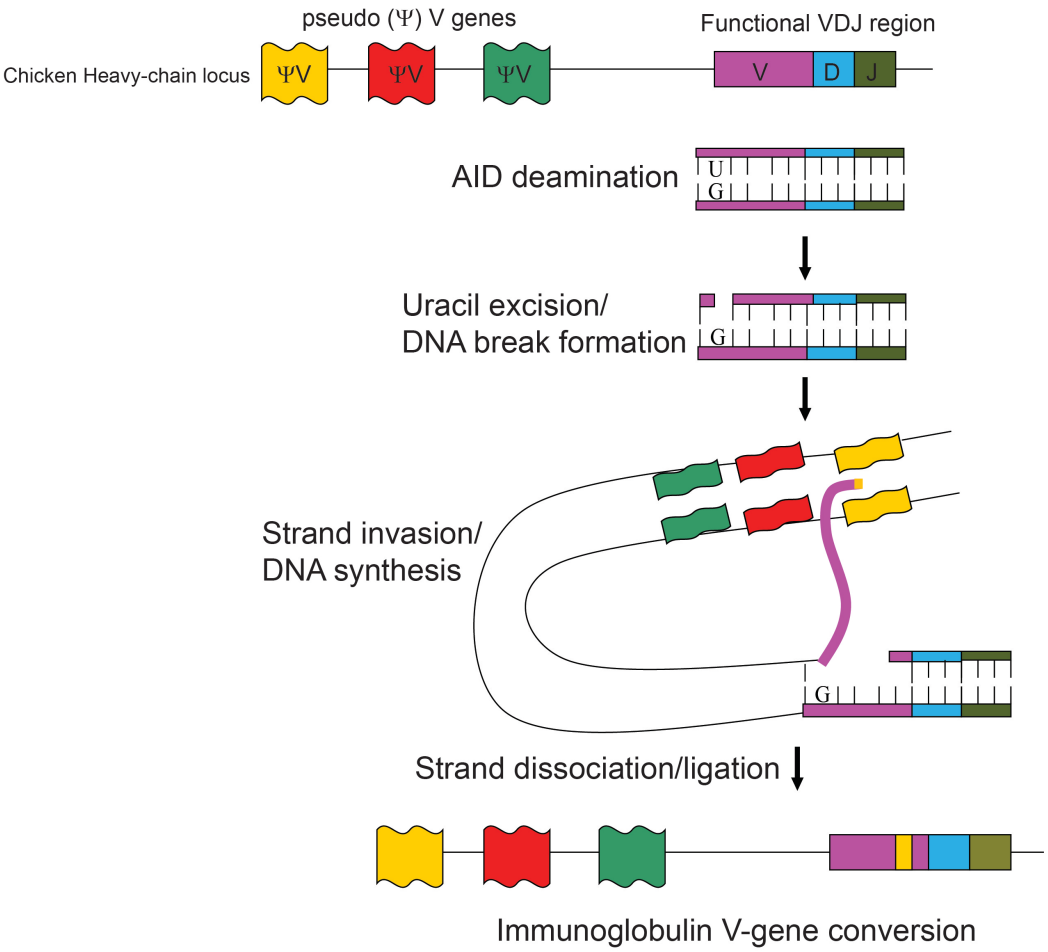

Figure 4. A possible model for Ig V-gene conversion. Birds generate one functional V(D)J segment and diversity is generated by gene conversion, i.e., the non-reciprocal transfer of DNA sequences from the upstream pseudo (Ψ) V-genes into the recombined V(D)J segment. DNA breaks are initiated by AID deamination of cytidine residues to uracils. The broken DNA strand invades upstream (Ψ) genes and DNA synthesis proceeds, followed by dissociation and annealing back to the original DNA region.

B-cell repertoire development occurs first in the yolk sac where V(D)J recombination takes place (37). Unlike the ordered V(D)J recombination observed in humans and mice, the chicken H-chain and L-chain undergo rearrangement at the same time (37). The bursa of Fabricius is the organ where Ig V-gene conversion is initiated in the absence of antigens (37). Antigen-dependent Ig V-gene conversion occurs in the germinal centers in the spleen where initially Ig V-gene conversion is high but later is it replaced by SHM (37).

2.2d Proteins involved in immunoglobulin V-gene conversion. Most of the proteins involved in Ig V-gene conversion are similar to proteins involved in gene conversion in yeast, although, there are major differences in the dependency for some of them as was discovered by gene knockout studies in the chicken B-cell line, DT40 (2). The enzyme AID is important for initiating DNA damage since Ig V-gene conversion is abolished when the AID gene (*Aicda*) is deleted (7). Uracils generated by AID deamination can be processed by the base excision repair (BER) protein, Ung, as Ung-deficient DT40 cells have reduced Ig V-gene conversion (38). The fact that Ig V-gene conversion is not abolished in Ung-deficient cells suggest that Ung-independent mechanisms may also play a role in removing the uracils. Removing of uracils by the BER pathway results in abasic sites that are usually targeted by apurinic/apyrimidinic endonucleases such as APE1 and APE2 that generate nicks in the DNA (4), however, it is not known whether they work in Ig V-gene conversion (39). The MRN complex, on the other

hand, is important as *Nbs1*$^{-/-}$ DT40 cells exhibit reduced Ig V-gene conversion (40-42).

Among the proteins that are needed to facilitate Ig V-gene conversion are Rad51 and the Rad51 paralogues, Xrcc2, Xrcc3, and Rad51B. Rad51 deletion in DT40 cells is lethal (43), however, deletion of Rad51 paralogues, *Xrcc2*$^{-/-}$, *Xrcc3*$^{-/-}$ and *Rad51B*$^{-/-}$ all result in a reduction of Ig V-gene conversion. Interestingly, these deletions caused an increase in SHM (44). This is in contrast to *Rad54*$^{-/-}$ DT40 cells, where only a reduction in Ig V-gene conversion is observed (45). These results suggest that the commitment to the Ig V-gene conversion pathway is regulated such that once homology search and strand invasion have begun, the decision to undergo Ig V-gene conversion cannot be changed. Hence in *Rad54*$^{-/-}$ cells, the broken DNA tails are already loaded with Rad51 and the scanning for homologous sequences has begun. Therefore, it is too late to initiate SHM. On the other hand, the Rad51 paralogues are needed for the initial steps in Ig V-gene conversion because they aid in the loading of Rad51 onto the broken DNA tails. Therefore, in their absence, increased SHM is observed because the cells have not yet committed to the Ig V-gene conversion pathway. Finally, removing all of the ΨV genes results in increased SHM, while deletion of proteins involved in non-homologous end-joining (NHEJ), for example Ku70 and DNA-PKcs, result in an increase of Ig V-gene conversion (46, 47). These results suggest that both HR and NHEJ pathways are functional in DT40 cells and that there may be competition between the two.

2.2e Immunoglobulin V-gene conversion in other species. Immunoglobulin V-gene conversion has been well documented in chickens as well as other avian species like quail, duck, pigeon, turkey, and hawk (48). Interestingly, Ig V-gene conversion is not avian specific in that it has been reported in mammals such as rabbits, cattle, swine, horses, and sheep (49). These species, unlike the chicken, do have more V(D)J genes that can be rearranged, but, not much diversity is generated by V(D)J recombination. For example, cows have about 20 V_H genes but they belong to the same gene family, and thus are highly homologous (49); rabbits have about 200 V_H genes but only one V gene, V_{H1}, closest to the D_H segment, is mostly rearranged (50); while in pigs, only 5 V_H genes comprise the majority of the pre-immune repertoire (51). Therefore, these species may use a combination of Ig V-gene conversion and SHM to further increase diversity of the B-cell repertoire.

It should be noted, however, that in some of the species, the evidence for Ig V-gene conversion is weak. For example, in rabbits, the non-reciprocal transfer of DNA segments, which defines gene conversion, has not been demonstrated, therefore, it is suggested that the diversification is by an Ig V-gene conversion-like mechanism (50). Similarly, it is disputed whether Ig V-gene conversion has a significant contribution for the generation of diversity for cows and pigs because not all of the V genes have been identified in these animals (49, 51).

Evidence of Ig V-gene conversion in humans and mice is lacking. Earlier reports in the 1980's and early 1990's have suggested that Ig gene conversion may be important in mice as sequences in hypermutating hybridoma cells were shown to match germline V gene sequences (52-54). However, these studies do not rule out the possibility that SHM may be responsible for their observation. Indeed, several studies have shown that SHM, rather than Ig V-gene conversion, is responsible for the diversifications observed in mice (55-58). The fact that gene conversion relies on a homology based mechanism, identifying gene conversion tracks among germline sequences is difficult. Furthermore, gene conversion can be confused with V_H-gene replacement, which is a type of B-cell receptor editing. This process changes the rearranged VDJ region by way of recombining with an upstream unarranged V-gene, resulting in the looping out parts of, or all of the original V-gene (Figure 5, (59)). This type of recombination relies on the Rag enzymes and cryptic RSS regions (cRSS) (60). The cRSS, which seem to be conserved in all jawed vertebrates (60), are similar to the heptamer sequence of the real RSS region but are located near the 3' end of many V_H elements (60). It has been argued that V_H replacements, observed in human B-cells, are actually Ig V-gene conversions because there is no evidence that Rag proteins are present, and further, the DNA junctions occur at AID hotspots (61).

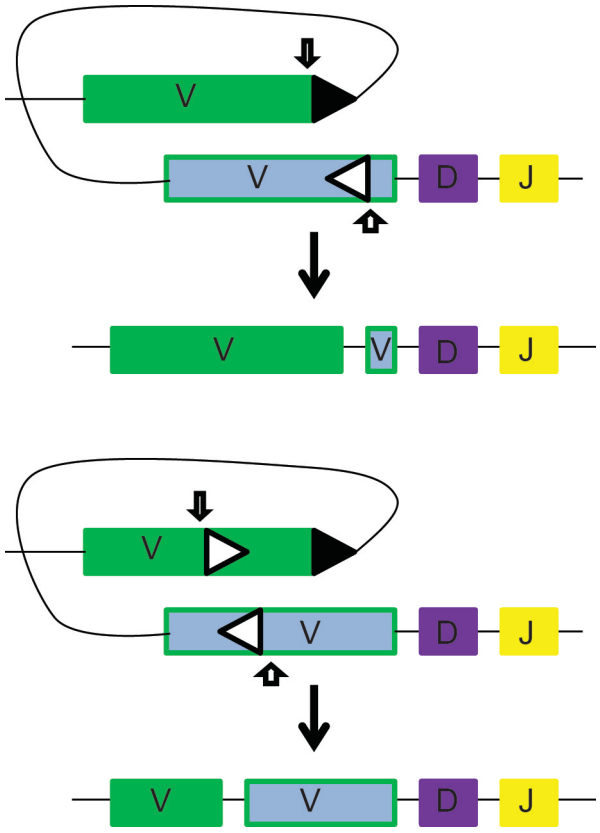

Figure 5. V_H-gene replacement. Changing the specificity of V_H regions can occur by replacing most, or part of, the V region with all, or part of, an upstream V gene. The Rag enzymes can recognize normal RSS regions (black triangle), composed of conserved heptamer and nonamer sequences separated by non-conserved spacer sequence or cryptic RSS regions (white triangle) which are usually located at the 3' region of V genes and are missing conserved heptamer and nonamer sequences. The recombination sites are depicted by small arrows.

Although evidence for the direct contribution of Ig V-gene conversion in the diversification of human and mouse B-cells is lacking, there is evidence that gene conversion may be indirectly involved in the diversification of mouse B-cells. It has been long known that proteins involved in gene conversion, like Rad51 and Rad52, are detected in B-cells (62, 63). However, it was not until recently that the contribution of gene conversion was shown to be important during B-cell diversification. Deletion of the *Xrcc2* gene, which aids in the loading of Rad51 onto 3' ssDNA tails, was shown to result in more DNA breaks (genomic instability), decreased CSR to IgG1, and proliferation defect (64). Taken together, this study suggests that by promoting genomic stability, gene conversion may play an indirect role during B-cell diversification of mice and humans.

2.2f Immunoglobulin V-gene conversion in transgenic mice. Transgenic mice designed to optimize the detection of Ig V-gene conversion have been previously generated in our laboratory. These transgenic mice, which contain two highly homologous recombined VDJ segments (2B4 and R16.7) that differ by 17 nucleotides have been shown to display gene conversion-like sequence transfers (65, 66). Only the downstream R16.7 VDJ region has a promoter and can be expressed. Expression of R16.7 VDJ region containing DNA segments from the upstream 2B4 VDJ region can only occur by gene-conversion-like sequence transfers because the 2B4 VDJ region is promotorless and cannot be expressed. Both high copy (VVCμ) and low copy (VV29) transgenic mice display

Ig V-gene conversions, although the estimated frequency at which the sequence transfers occur seem to be fairly low as antigen selection increases their detection (65-67). These results suggest that the gene conversion machinery is functional during mouse B-cell activation. The mechanism may not be the same as in chickens because Rad54 was shown not to be required in mice (68). One of the aims of this thesis is to further investigate whether transgene Ig V-gene conversion is similar to chicken Ig V-gene conversion in requiring AID.

2.3 Somatic hypermutation

Somatic hypermutation (SHM) is a B-cell diversification mechanism that introduces point mutations into recombined V(D)J regions during B-cell activation, and thereby, can lead to selected increases in the affinity of antibodies during responses to pathogens (69, 70). AID initiates SHM by deaminating cytosine residues to uracils within WRC (W= A/T, R=A/G) motifs that are AID hotspots (29, 71). High levels of transcription are required (72, 73) and mutations typically start 100-150 bp downstream of the V-gene promoter, extending about 1.5-2 kb further down (74-78). The highest frequency of mutations are in the V(D)J and intronic J regions, ending before reaching important regulatory *cis* elements and the C regions (Figure 6, (79, 80)). Although, most heterologous promoters that can activate high rates of transcription can substitute for the Ig promoter (81, 82), the position of the V-gene promoter seems to be critical in targeting SHM to the V(D)J region. For example, placing a V-gene promoter upstream of the Cκ gene caused mutations

within the normally unmutated Cκ region, with similar dependence on the distance from the promoter (79). The rate of SHM is about 10^{-3} to 10^{-5} mutations per cell cycle, which is close to a million fold higher than the spontaneous mutation rate that occurs in the rest of the genome (83, 84).

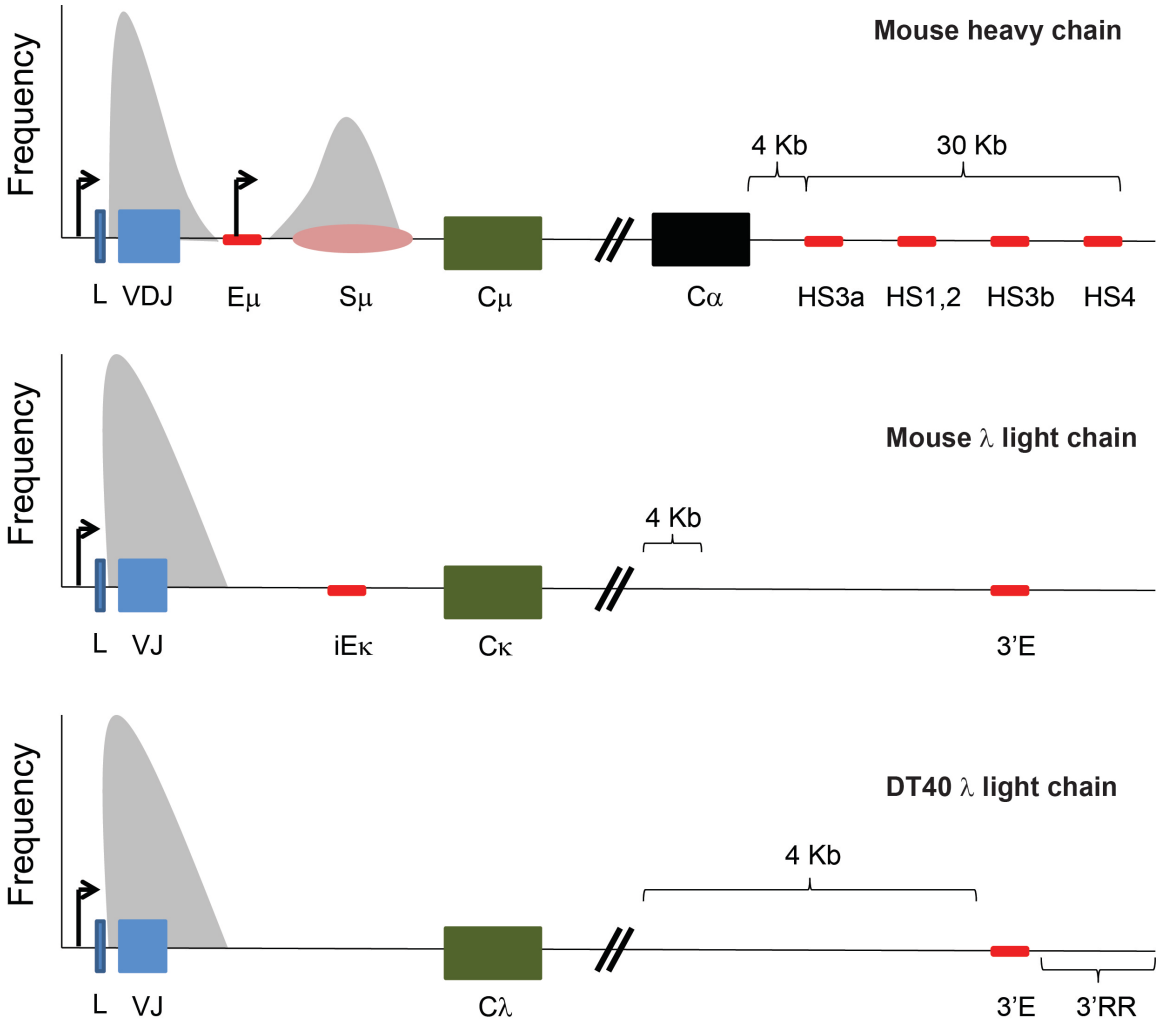

Figure adapted from Maul RW and Gearhart PJ. *Adv Immunol*. 2010

Figure 6. Somatic hypermutation frequency. The regions of high mutation frequency are depicted by gray curves. Promoters (arrows), E: enhancers, C: constant genes, Sμ: switch μ region, HS3a, HS1,2, HS3b, and HS4 are the DNase I hypersensitive regions located about 4 kb downstream of the Cα gene. This region, which is about 30kb, is called the 3' regulatory region (3'RR) and has enhancer activity. DT40 is a chicken lymphoma cell line. Diagram not drawn to scale.

2.3a The molecular mechanism of somatic hypermutation. Activation-induced cytidine deaminase targets both the transcribed (template, bottom) and the nontranscribed (nontemplate, top) DNA strands and generates U:G bp mismatches (3). If the uracil is not removed from the DNA upon replication, the general replication machinery will mistake the uracil with thymidine and replicate over the uracil generating C to T transition mutations in one of the daughter cells (85, 86). More frequently, however, the base excision repair (BER) and mismatch repair (MMR) pathways play an important role in processing the U:G bp mismatches, which results in mutagenesis at the Ig locus (Figure 7). However, the conventional function of these repair pathways is to promote error-free repair and protect the genome against deleterious mutagenesis (86-88).

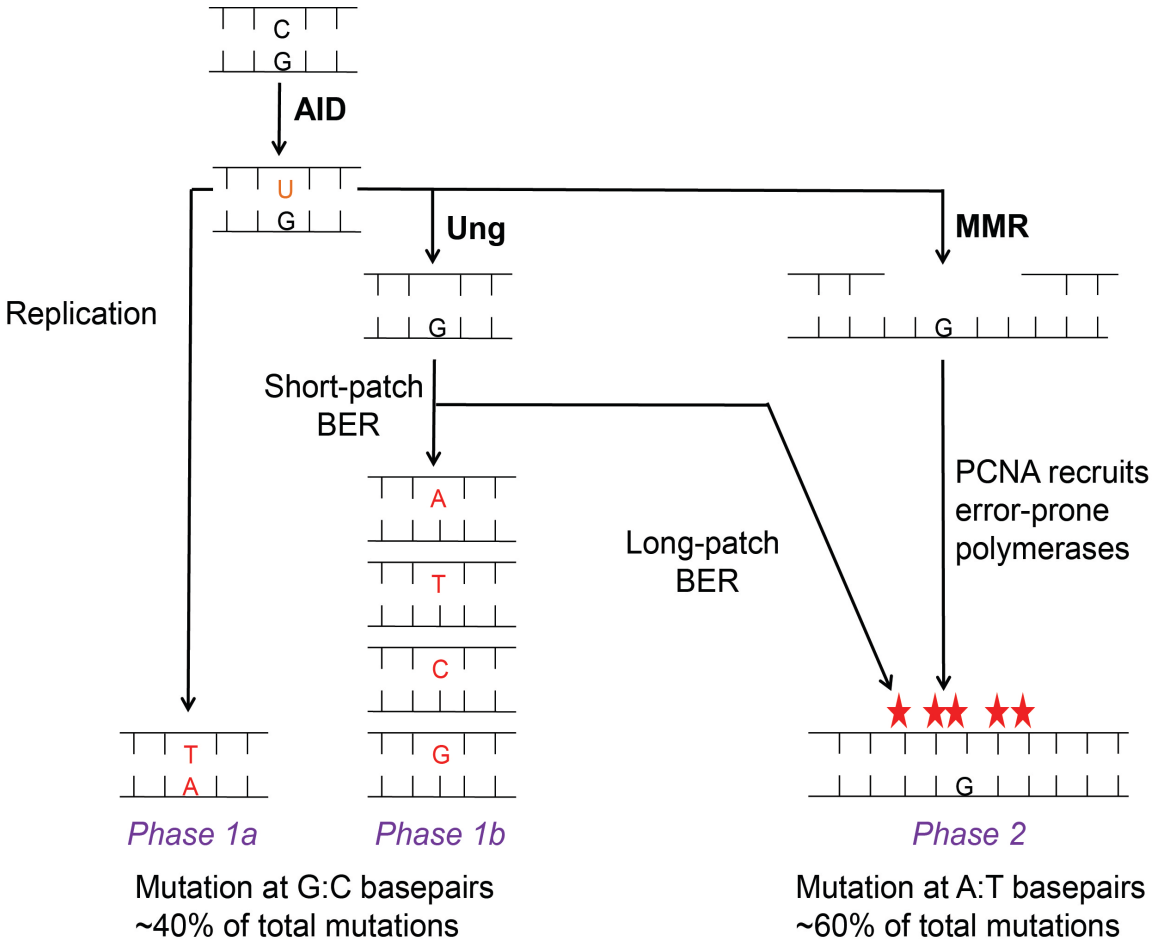

Figure adapted from Peled JU et. al. *Annu. Rev. Immunol*. 2008

Figure 7

Figure 7. BER and MMR pathways during SHM. Deamination of cytosine (C) to uracil (U) by AID leads to DNA damage that can be repaired by several pathways. If the U is not removed, the DNA replication machinery can mistake the U for a deoxythymidine (T) and one of the daughter cells can acquire a C to T transition mutation (Phase 1a). If the U is removed, the abasic site can be repaired by short-patch base excision repair (BER) and the gap filled in with error-prone DNA polymerases, inserting any nucleotide in place of the U (Phase 1b). The U, and surrounding sequences, can also be removed by mismatch repair (MMR) pathway, followed by monoubiquitination of PCNA and repair by error-prone DNA polymerases. MMR and long-patch BER can generate mutations at base pairs surrounding the original C residue that was deaminated by AID (Phase 2).

Base Excision Repair

Removal of uracils by Ung, which is part of the BER pathway, creates an abasic site (3). The abasic site can be further converted to single-stranded DNA (ssDNA) nicks. The obvious candidates for the generation of ssDNA nicks are the purinic/apyrimidinic (APE1/APE2) endonucleases that have been shown to be important in CSR (89) and in error-free DNA repair (90). However, it is not known if APE1/APE2 function in SHM. In fact, studies have shown that the MRN complex can cleave DNA at the abasic sites (40, 91, 92). Nevertheless, both the abasic sites and ssDNA nicks are filled in by error-prone DNA polymerases that can result in the generation of both transition mutations (purine to purine or pyrimidine to pyrimidine) and transversion mutations (purine to pyrimidine) due to the lack of proofreading activity of the error-prone DNA polymerases (3). Because *Ung*$^{-/-}$ B-cells lack most G/C transversion mutations and B-cells deficient in error-prone DNA polymerase Rev1 display decreased frequency of G/C transversion mutations, it has been suggested that Rev1 functions downstream of Ung (85, 93).

Mismatch Repair

As part of the mismatch repair (MMR) pathway, the Msh2 and Msh6 heterodimers can also recognize U:G bp mismatches and initiate the removal of the uracils by DNA excision followed by resynthesis via error-prone polymerases that can produce mutations at base pairs surrounding the original C:G site that was deaminated by AID (87, 88, 94). Similar to the conventional function of the

BER pathway, the MMR pathway is also important in protecting the genome from deleterious mutagenesis, but at Ig loci, it has a mutagenic function (95). The pathway is initiated by the binding of Msh2/Msh6 heterodimer to the mismatch, which leads to the recruitment of Mlh1/Pms2 or Mlh1/Mlh3 and the generation of ssDNA nicks near the mismatch (3). The exonuclease Exo1 then excises the mismatch, including a section of the DNA strand containing the mismatch, leading to the recruitment of error-prone polymerases for resynthesis of the DNA strand and ligation by DNA ligase I (3). Deletion of Msh2, Msh6, or Exo1 result in about 80-90% reduction in mutations at A/T base pairs (87, 88, 96). This indicates that majority of A/T bp mutations are attributed to the MMR pathway. The rest of the A/T bp mutations are attributed to Ung, as *Msh2/Ung* double knockout B-cells display a complete absence of A/T mutations (86). Similarly, deletion of the error-prone DNA polymerase eta (η) result in almost a complete lack of mutations at A/T base pairs, implying that Pol η functions downstream of BER and MMR during SHM (97-100).

As the result of the different DNA repair pathways involved downstream of AID deamination, about 60% of the mutations are at A/T base pairs, of which 50% are transversions. The other 40% of the mutations occur at G/C base pairs (101). This indicates that majority of the mutations generated during SHM are not due to direct AID activity but rather due to the repair mechanism downstream of AID.

2.3b Regulation of somatic hypermutation. The DNA repair pathways that function during SHM are evolutionary conserved to protect the genome against deleterious mutagenesis. Exactly how they become mutagenic at the Ig locus during SHM is not clear. Obviously, error-prone DNA polymerases play an important role in the mutagenesis process. Error-prone DNA polymerases are highly mutagenic when replicating across undamaged DNA and abasic sites (102). For example, at abasic sites, the error-prone DNA Pol η preferentially inserts mismatch nucleotides opposite template thymidine (T) (103). Nevertheless, error-prone DNA polymerases have biological significance, as indicated by the hereditary disease Xeroderma pigmentosum (XP) where the absence of Pol η causes sunlight sensitivity and predisposition to skin cancer (104, 105).

Several of error-prone DNA polymerases, Pol η, Rev1, and to some degree Pol κ, have been implicated in SHM. Each error-prone DNA polymerase has an unique mutagenic signature and alterations in the mutation spectrum can be attributed to the absence of specific polymerases. A decreased frequency of mutations at A/T bp is observed in Pol $\eta^{-/-}$ B-cells (97-99), while a decrease in the frequency of G/C to C/G transversions is dectected in Rev1$^{-/-}$ B-cells (93). DNA Pol κ is responsible for A/T mutations in the absence of Pol η (106, 107). So, it would make sense to suggest the DNA repair pathways during SHM become mutagenic because they recruit these error-prone DNA polymerases for the DNA resynthesis step. This explanation, however, does not address the fact that

during B-cell activation, error-free DNA polymerases, such as Pol β, are also functional at the Ig locus (108), and there seems to be no evidence of specific upregulation of error-prone DNA polymerases during SHM. Moreover, *ex vivo* and *in vivo* experiments have shown that physiological levels of AID can target multiple chromosomes throughout the genome during B-cell activation (109, 110). These studies show that both error-free and error-prone factors are present during B-cell activation, and that the DNA repair pathways can have both mutagenic or nonmutagenic function depending on the chromosomal location where the DNA damage occurred. The mechanism(s) that govern mutagenesis at one locus and error-free repair at another locus are not very clear. However, some very elegant studies in yeast have shown that post-translational modification of proliferating cell nuclear antigen (PCNA) is important in loading of error-prone DNA polymerases (111-113).

Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA) is a ring shaped homotrimer, which encircles the DNA and is able to slide back and forth, and is often referred as the master regulator of both DNA replication and repair due to its ability to recruit several different proteins to the DNA template (114). It is essential in the processivity of error-free DNA polymerases δ and ε, and thus plays an important role during DNA replication by tethering polymerases to the DNA template (114, 115). PCNA also interacts with DNA repair proteins such as MSH6, EXO1, MLH1, UNG, and many others, therefore, having a vital role in BER and MMR

pathways (114). The ability of PCNA to interact with both error-free and error-prone DNA polymerases was shown to dependent on the ubiquitination status of PCNA as monoubiquitination increased the affinity of PCNA for loading the error-prone DNA Pol η (113, 116, 117). More specifically, monoubiquitination of PCNA at lysine 164 (K164) is essential in the loading of error-prone DNA polymerases (112, 118, 119). Studies in mice that express a mutated form of PCNA which cannot be monoubiquitinated ($PCNA^{K164R}$) show a drastic reduction in mutations at A/T base pairs during SHM (120, 121). PCNA monoubiquitination is required for A/T mutations downstream of both Msh2 and Ung (122). Furthermore, defects in PCNA monoubiquitination in DT40 B-cell lines result in decreased SHM (123).

Studies in yeast have shown that monoubiquitination of PCNA at K164 is achieved by the ubiquitin-activating enzyme E1, the E2-ubiquitin-conjugation enzyme RAD6, and the E3 ubiquitin ligase RAD18 (111). This constellation of molecules is referred to as the RAD6 pathway and is implicated in controlling polymerase switching and error-prone DNA repair as RAD18 is needed for Pol η to interact with PCNA (119). In mouse embryonic stem cells, deletion of RAD18 results in genomic instability, while a 3-fold decrease in SHM is observed in RAD18-deficient DT40 chicken B-cell lines (124, 125). RAD18 is also required for gene conversion in DT40 cells (126).

Polyubiquitination of PCNA is thought to be important in error-free repair (113). Here, the polyubiquitin chains are linked via K63 of ubiquitin, and therefore, is different from the K48-linked polyubiquitination which usually targets proteins for proteosomal degradation (111, 127). In yeast, polyubiquitination of PCNA is dependent on the heterodimeric E2 ubiquitin conjugases Ubc13 and Mms2, and the E3 ubiquitin ligase Rad5 (113). Studies in mice where the Rad5 orthologs, HLTF and SHPRH, were deleted revealed no effect on SHM (128). Furthermore, SHM in DT40 chicken B-cell lines does not depend on Ubc13 (129). These results suggest that the regulation of PCNA ubiquitination in higher eukaryotes are different from yeast.

p21 and p53

The regulatory mechanisms that promote error-prone repair by monoubiquitinating PCNA in B-cells are not yet known. Studies in human cell lines have shown that the tumor suppressor protein p53, and the cell cycle inhibitor protein p21 (also known as CDKN1A and $p21^{Cip1/Waf1}$) control mutation loads by affecting PCNA ubiquitination (130, 131). Because p21 binds PCNA near the vicinity of the ubiquitination site, it has been suggested that p21 binding to PCNA may impair the ability of PCNA to switch to a lower fidelity DNA polymerase such as Pol η (131-133). Indeed, increased levels of Pol η foci formation on DNA and increased Pol η/PCNA interactions have been observed in *p21*$^{-/-}$ cells lines (118, 133). These results indicate that p53 and p21 function together to protect the genome from deleterious mutagenesis.

The functions of p21 are many and varied. Expression of p21 is directly induced by p53 (134). As a member of the cyclin-dependent kinase (CDK) inhibitors, p21 regulates the cell cycle by binding to both cyclins and CDKs, and thus, inhibiting their ability to phosphorylate the retinoblastoma (Rb) protein. This modification allows the cells to proceed through the G1 phase of the cell cycle (135). The N-terminus region of p21 interacts with CDKs (136, 137), while the C-terminus region interacts with PCNA (132, 138). It has been shown that p21 can both inhibit the cell cycle (139-141), and promote cell cycle progression depending on the specific cyclin/CDK complexes with which it is associated (142, 143). Furthermore, p21 can inhibit PCNA-dependent DNA replication by blocking the binding of PCNA with DNA Pol δ (144-146).

Many types of cellular stresses, such as DNA damage, hypoxia, and chromosomal aberrations can activate p53 (147, 148). The role of p53 during BER is well established in many types of cells (149-153). Furthermore, p53 can activate the transcription of the MMR protein MSH2 during DNA damage, indicating that p53 plays an important role during DNA damage repair (154). The roles of p21 and p53 during B-cell activation, however, are not so clear. Studies in B-cell lines have suggested that inactivating p21 during B-cell activation may be essential to allow proper cell cycle progression (155, 156). Specifically, it has been suggested that both p21 and p53 are not present in activated B-cells due to the presence of the transcriptional repressor BCL-6 which negatively regulates both p53 and p21 (156, 157). These studies argue that because activated B-

cells will acquire significant amounts of DNA damage during SHM and CSR, repression of p21 and p53 is essential to prevent cell cycle arrest and promote B-cell proliferation.

In support of this idea, another study in primary mouse B-cells showed that p21 is cleaved upon B-cell stimulation by caspase 3, although, in this study, the presence of p21 did not cause cell cycle arrest, but rather, increased proliferation (155). Contrary to these results, however, low levels of p53 protein have been detected in activated B-cells (158). Furthermore, the SHM spectrum is altered in B-cells of young (2 month old) *p53*$^{-/-}$ mice, while the SHM frequency is progressively decreased as p53$^{-/-}$ mice get older (3-4 months) (159) suggesting that, aside from controlling the cell cycle, p53 may regulate mutagenesis during SHM.

For CSR, p53 has been shown to inhibit isotype switching to IgG2a and to decrease DNA breaks and mutagenesis at Ab switch (S) regions (160). This function of p53 has been attributed to the ability of p53 to regulate the levels of intracellular reactive oxygen species (ROS). The authors in this study argue that p53 plays a protective role in B-cells by limiting AID activity. Indeed, p53 has been shown to prevent AID-induced interchromosomal translocations involving c-myc and Igh regions which are the hallmark of Burkitt's lymphomas (161). Although, p21 has not been implicated in preventing c-myc/Igh transloctions (162), due to the ability of p21 to bind and modulate PCNA monoubiquitination in

human cell lines (131-133), it is possible that p21 may also regulate mutagenesis in B-cells during SHM. As part of this thesis, the role of p21 in SHM has been investigated.

2.3c Targeting of somatic hypermutation. The potential ability of AID to cause genome-wide mutagenesis suggests that proper targeting of AID to the Ig locus is essential in preventing deleterious mutagenesis. Sequences composed of regulatory elements such as Ig enhancers and promoters located at Ig loci have been implicated in AID targeting. Recently, the existence of targeting elements for SHM and Ig V-gene conversion within the endogenous Ig locus were identified in DT40 chicken cell lines. The investigators identified a 9.8 kb region, downstream of the L-chain transcriptional start site, that is required for SHM (163, 164). Studies in mice, have shown that transgene reporters on almost any chromosomal location can become hypermutated as long as the transgene contains a strong promoter, the intronic L-chain enhancer (iEκ), and 3' L-chain enhancer (3'Eκ) (Table 1; page 57, (165)). Furthermore, an Ig transgene containing bacterial sequences in the place of V-regions can be subjected to SHM as long as the transgene contains the appropriate L-chain *cis* element discussed above (166).

Surprisingly, deletions of the endogenous L-chain enhancers at the κ locus, did not have much effect on SHM (167, 168). Furthermore, replacing the chicken L-chain enhancers with the mouse L-chain enhancers supported

transcription but not SHM in the chicken DT40 cell line, indicating that regions surrounding the L-chain enhancers may be important for efficient targeting of SHM to Ig regions (169). Alternatively, it is also possible that targeting of SHM is species-specific (169).

Regulatory enhancers at the H-chain are the intronic Eμ (Eμ) enhancer and the 3' regulatory region (3'RR) enhancers. The Eμ enhancer, located downstream of the J_{H4} segment was discovered 30 years ago as the first Ig transcriptional enhancer (170-172). Eμ was shown to be required for D_H to J_H recombination and deletion resulted in disruption of VDJ recombination, however, SHM was not significantly affected (Table 1; page 57, (173, 174)). A study in Ramos B-cell lines, however, showed a 2-fold reduction in SHM if the Eμ was deleted on an Ig transgene (175). These observations suggest that Eμ and surrounding sequences may make SHM more efficient.

The 3'RR enhancers, spanning about 30 kb downstream of the Cα gene, consist of four DNase I-hypersensitive sites (HS3a, HS1,2, HS3b, HS4) whose functions have been investigated in knock-out studies (Table 1; page 57, (165)). Three additional regions, HS5, 6, and 7 have also been identified, however, their role in Ig diversification has not been determined yet (3). Immunoglobulin transgenes that are missing 3'RR enhancers can undergo SHM after translocation into the endogenous Igh locus (176, 177). These studies indicate that SHM may require the presence of 3'RR enhancers. Studies, have shown

that, while HS3b and HS4 are sufficient to target SHM to Ig transgenes, HS1,2 are not (178, 179). At the endogenous Igh locus, however, deletions of both HS3b and HS4 (*Hs3b*$^{-/-}$:*Hs4*$^{-/-}$ double knockout) does not affect SHM (180). These studies indicate that the 3'RR enhancers may work synergistically to achieve a strong enhancer effect. Evidence of this synergistic function comes from studies that show that SHM is significantly decreased when all the 3'RR enhancers are deleted on an Ig transgene (181). It should be noted, however, that because deletions of the 3'RR enhancers also disrupt transcription at the Ig locus, it becomes difficult to distinguish loss of transcription with loss of targeting.

Additional elements that may be important for targeting SHM are E-box motifs which can target a variety of transcription factors (182). Immunoglobulin L-chain transgenes with E-box motifs show an increase in SHM frequency (182). Furthermore, histone modifications associated with transcriptionally active chromatin also play a role in AID targeting (3). However, because these sequences and modifications are also present in many non-mutated genes, it is unclear whether they play a direct regulatory role in AID targeting (3).

2.3d Limiting activation-induced cytidine deaminase activity. The mutagenic activity of AID could be detrimental to cells if not regulated properly. Therefore, limiting AID activity would be a good start in controlling genome-wide mutagenesis. Transcription of AID is induced within one day post B-cell activation (183). Only about 15% of AID protein is in the nucleus; the rest is in

the cytoplasm (184). Furthermore, only phosphorylated AID is present in the nucleus and phosphorylation at serine 38 (S38) seems to be important for SHM and CSR (184-187). Phosphorylation can also limit AID activity. For example mutation of S3 to alanine (S3A) prevents phosphorylation at this residue, and also increase SHM, CSR, and c-myc/Igh translocations (188). Two miRNAs, miR155 and miR181b, have been shown to negatively regulate AID mRNA levels (189-191). Studies from AID mutants have shown that the N-terminus of AID is essential for SHM while the C-terminus of AID is important for CSR (192-194). Finally, AID has been shown to interact with a variety of different proteins which may regulate AID targeting, however, because these AID interacting partners also function at non-Ig loci, protein(s) that specifically targets AID to the Ig locus have not yet been identified (195).

2.4 Class Switch Recombination

Class switch recombination (CSR) is a process that switches Ig H-chain constant (C_H) regions, thereby, altering the Ig protein effector functions. This produces different classes, also known as isotypes, such as IgG, IgA, IgE, without changing the Ab binding sites (VDJ regions).

2.4a Isotype effector function. There are eight C_H regions, or classes of antibodies, in most inbred mice used in the laboratory. They are organized as 5'-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3' (4). The IgD (Cδ) isotype is corexpressed

with IgM (Cμ) and can be expressed in the absence of antigenic stimulation by a mechanism that involves alternative RNA splicing of the primary transcript that contains both Cμ and Cδ (196). Each isotype has a specific effector function which is important in determining how pathogens are removed from the body. For example, IgM antibodies, which are present prior to CSR, form pentamers which increase their avidity (combined synergistic strength) against multimeric antigens during the early immune response (197). Isotypes that are generated post-CSR can bind via their Fc regions to Fc receptors (FcR) that are expressed on many different immune cells. Examples include the Fcγ receptor II (FcγRII) and FcγRIII that are expressed on mast cells, where IgG1 (Cγ1) binding to the receptors can result in mast cell degranulation and increase vascular permeability that can aid in initiating an effective immune response against nematode parasites (198, 199). Similarly, IgG2b (Cγ2b) can bind to FcγRII or FcγRIII on macrophages and natural killer (NK) cells and aid in removing viruses and gram-negative bacteria from the body (200, 201). IgG isotypes can also cross the placenta and provide protection for the fetus (202). The IgE (Cε) antibodies can bind to mast cells, basophils, and eosinophils via the FcεR (203) which may play an important role in removing parasites and viruses (198, 204-206), while IgA (Cα) antibodies are important in protecting against gut microbes and viruses at mucosal sites (207). Lastly, the isotypes, IgG1, IgG3, and pentameric IgM are also able to activate the complement system that aids in the removal of antigens from the body (208-210).

2.4b The mechanism of class switch recombination. The mechanism of CSR involves deletional recombination events between non-homologous switch (S) region DNA sequences located upstream of each C_H gene. The recombination event occurs by intrachromosomal joining between the Sμ region to one of several downstream S regions located on the same chromosome (4), Figure 8).

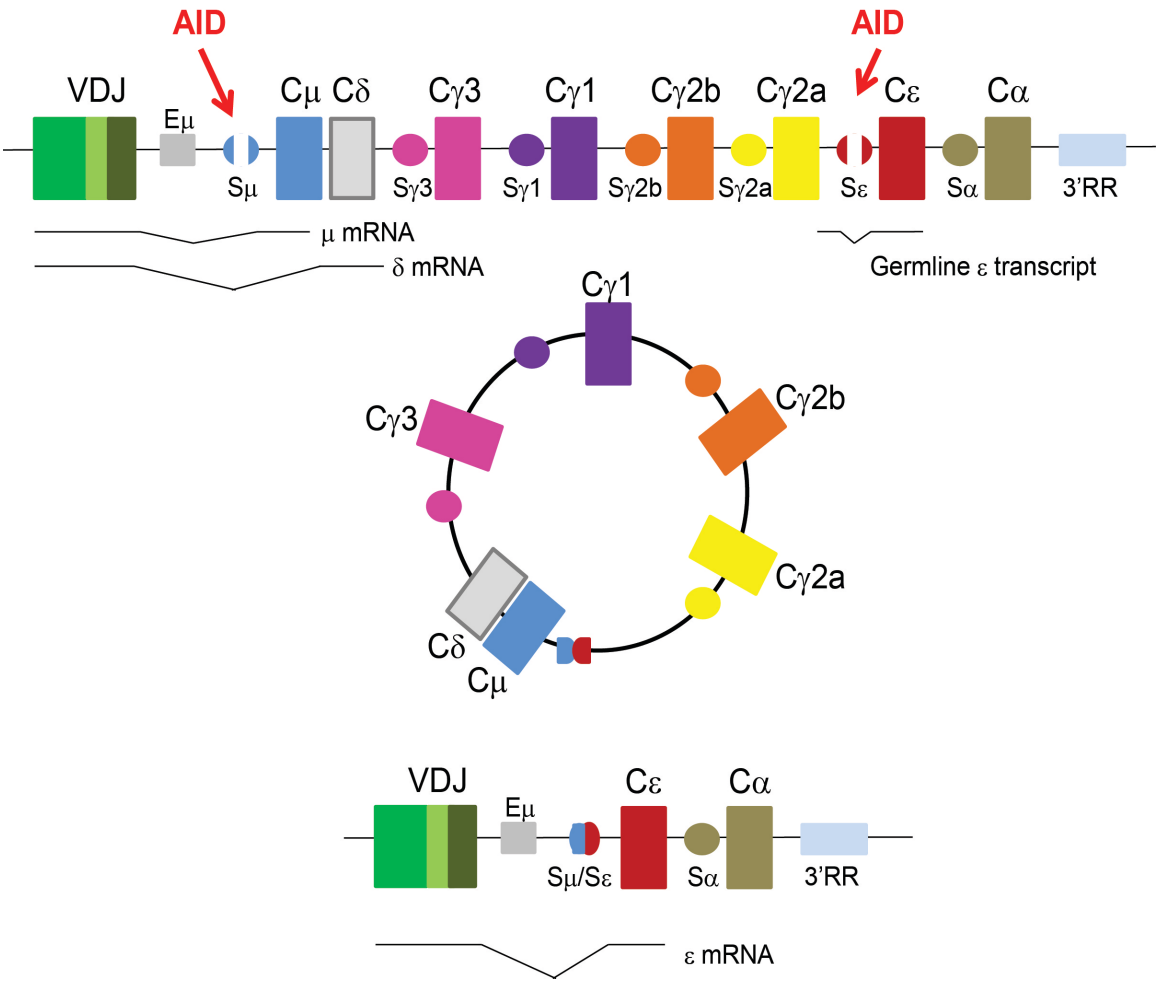

Figure adapted from Stavnezer J. *Trends in Immunology* 2011

Figure 8

Figure 8. Molecular mechanism of CSR. Class switch recombination (CSR) is initiated by AID deamination of cytosine residues at switch (S) region sequences which result in double stranded breaks. Germline transcription is needed to target and provide ssDNA substrates for AID. The Sμ region is transcribed in the absence of mitogen stimulation, while transcription of S regions downstream of Sμ are activated by isotype-specific mitogens. In the figure, switching to Cε is depicted. Upon joining of Sμ and Sε, the intervening DNA is looped out and excised, bringing the recombined VDJ region close to the Cε constant gene.

Switch regions

Each S region consists of tandem repeats of short (20-80 bp) G-rich sequences that vary in length from 1 kb-12 kb (211). These repetitive S regions contain abundant WGCW motifs (W=A/T), which are the hotspot motifs that are recognized by AID (212). The Sμ region is the most repetitive and contains the highest number of AID hotspots (213, 214). Some sequence homology exists between Sμ, Sε, and Sα regions, however, there is no homology between the Sμ and Sγ regions (214). With exception of Sμ, recombination sites are found anywhere within the S region tandem repeat (TR) sequences . For Sμ, about 80% of the S junctions are within the SμTR, while about 20% occur 5' of the SμTR (215). S regions are not, however, recombined in regions of long lengths of homology, but rather, most switch junctions appear to result from joining switch region DNA breaks without homology or with short stretches of microhomology (216). Studies from knockout mice support the hypothesis that S regions are important for CSR. For example, CSR is reduced in mice that lack the Sμ region (217, 218), suggesting that the Sμ region is required for efficient CSR. CSR, however, is completely blocked in B-cells lacking the Sγ1 region (219). These studies suggest that the regulatory mechanism(s) in the Sμ regions are different from downstream S regions.

Germline transcription

CSR is regulated by isotype-specific activation of transcription. Transcription is initiated from the intronic (I) promoter, located upstream of every S region.

These transcripts, containing the non-coding I exon, the intronic S region, and the associated C region exons, are termed germline (GL) transcripts because they do not code for a functional protein (220, 221). The rate of CSR seems to correlate with the rate of transcription (222), and GL transcription is required for CSR as shown by the deletion of Iγ1 and Iγ2b sequences which impaired switching to Cγ1 and Cγ2b, respectively (223, 224).

Stimulation of B-cells with isotype-specific mitogens and cytokines induces transcription of specific GL promoters at different S regions, and thus, directs CSR to specific isotypes (221). For example, B-cell activation that requires the help of T-cells (T-cell-dependent (TD) mechanism) involves interaction of CD40 on B-cells with CD40 ligand on T cells, which can be mimicked by the addition of anti-CD40 antibodies in B-cell cultures. While T-cell independent (TI) activation of B-cells can be mimicked by stimulating B-cells with bacterial lipopoly-saccharide (LPS). More specifically, LPS stimulation in combination with specific cytokines can lead to CSR to specific isotypes (225-230). It should be noted, however, that expression of Iμ-Cμ GL transcription is constitutive and is not affected by cytokine stimulation (231).

GL transcription appears to be important for targeting AID to S regions through direct interaction of AID with the transcriptional machinery, as it was shown that AID is co-immunoprecipitated with RNA polymerase II (232). Furthermore, transcription might alter histone modifications which may target AID

to the S regions (4, 233-235). GL transcription may also provide the ssDNA substrates for AID. The transcriptional bubble that is formed during transcriptional elongation may expose ssDNA substrates for AID (4). Furthermore, during transcription through the G-rich S regions, GL transcripts form RNA-DNA hybrids (R-loops) with the bottom C-rich DNA template strand, leaving the top G-rich DNA strand single stranded, and thus, providing the ssDNA substrates for AID (236). R-loops, however, may only make CSR more efficient as CSR was only slightly decreased when the G:C-rich mammalian Sγ1 region was substituted with the A:T rich frog Sμ region which does not form R-loops (237).

As R-loops and transcriptional bubbles expose the top DNA strand for AID deamination, antisense transcription at S regions may make AID accessible at bottom DNA strands. While bidirectional transcription of S regions have been detected (238), there is evidence that, at least for the Sγ3 region, antisense transcription is largely dispensable for CSR (239). Alternatively, splicing of the GL transcript may collapse the R-loops leaving the bottom DNA strand exposed (240). In this context, it was found that CSR to IgG1 was inhibited when the donor splice site of Iγ1 was mutated (241). Nevertheless, it is not known whether splicing has any role in making the bottom DNA strand exposed to AID (240). Recently it was shown that AID can associate with a component of the RNA exosome (242) which removes nascent transcripts from transcribed DNA. They

authors in this study proposed that association of the RNA exosome with AID and RNA Pol II may exposing the bottom DNA strand for AID.

Enhancer elements

Similar to SHM and V(D)J recombination, *cis* elements play an important role during CSR. Deletion of the Eμ intronic enhancer region decreased CSR by about two-fold, although GL Sμ transcription was not eliminated (Table 1, (243)). Similarly, deletion of individual 3'RR enhancers from the endogenous Igh locus (*Hs1,2*$^{-/-}$, *Hs3a*$^{-/-}$, *Hs3b*$^{-/-}$, *Hs4*$^{-/-}$) does not affect CSR (244-246). However, combined deletion of all the 3'RR enhancers, both at the endogenous locus and on a transgene, had a dramatic effect on CSR to all isotypes (181, 247, 248). Furthermore, deletion of all 3'RR enhancers at the endogenous locus resulted in a dramatic decrease in GL transcription of all isotypes, including Igμ (247). Interestingly, replacing the HS1,2 enhancer with a neo^{R} cassette resulted in disruption of GL transcription and CSR to Cγ3, Cγ2a, Cγ2b, and Cε (244, 249), while a clean deletion of the HS1,2 enhancer (*Hs1,2*$^{-/-}$) had no effect on CSR (244). These results suggest that disrupting the higher order chromosomal structure of the Igh locus, i.e. by inserting non-Ig DNA sequences, can affect CSR perhaps by preventing proper function of the Igh enhancer elements. Indeed, chromosome conformation capture techniques have revealed that the 3'RR enhancers interact with the Eμ enhancer regions, providing physical evidence for the role of the 3'RR in CSR (250). Furthermore, according to reporter gene experiments, 3'RR and Eμ enhancers have synergistic

transcriptional activity (251, 252). These results indicate the Igh enhancers are important for CSR, probably by promoting high rates of transcription and facilitating the proper chromosomal conformation needed for efficient CSR.

Table 1	CSR	SHM	V(D)J
Endogenous Igh locus			
HS3a $^{-/-}$	no phenotype [a]		
HS1,2 $^{-/-}$	no phenotype [a]		
HS1,2 or HS3a replaced with neo^{R} cassette	CSR and transcription to Cγ3, Cγ2a, Cγ2b, Cε drastically decreased [a, b]		
HS3b $^{-/-}$	no phenotype [c]		
HS4 $^{-/-}$	decrease membrane IgM [d]		
HS3b $^{-/-}$:HS4 $^{-/-}$	Severe decrease in CSR and transcription to all isotypes except Cμ and Cγ1 [f]	no phenotype [k]	no phenotype [k]
Deletion of all four HS sites (HS3a:HS1,2:HS3b:HS4)	Severe defects in CSR and transcription to all isotypes [g]		
Eμ $^{-/-}$	2-fold decrease [o]	no significant effect [n]	impaired [n,m]
Igh Transgene			
Deletion of all four HS sites (HS3a:HS1,2:HS3b:HS4)	Severe defects in CSR and transcription to all isotypes [h, i]	decreased SHM as compared to wiltype transgene [h, i]	
addition of HS1,2		10-fold lower mutation and decreased transcription [j]	
addition of HS3b and HS4		mutation frequency comparable to endogenous V gene [l]	
Endogenous Igκ locus			
3'Eκ $^{-/-}$		no phenotype [p]	
iEκ $^{-/-}$		no phenotype [q]	
Igκ Transgene			
addition of iEκ and 3'Eκ		required [r, s]	
Igλ Transgene			
addition of Igλ 2-4 enhancer		low levels of SHM [t, u]	

Table 1. The importance of immunoglobulin *cis* elements in mice.

References: [a](244), [b](249), [c](245), [d](246), [e], [f](253), [g](247), [h](181), [i](254), [j](179), [k](180), [l](178), [m](173), [n](174), [o](243), [p](168), [q](167), [r](81), [s](255), [t](256), [u](257).

2.4c DNA repair pathways in class switch recombination. Similar to SHM, S regions deaminated by AID are processed by the BER and MMR DNA repair pathways which lead to the generation of DNA breaks within the donor Sμ region and an acceptor Sx region (x being any S regions downstream of Sμ). The initial removal of uracils from DNA generates single-stranded DNA breaks (SSBs) that must be further processed to generate the double-stranded DNA breaks (DSBs) needed for the recombination process. However, processing of the SSBs to generate DSBs is not necessary if the SSBs are relatively near each other, on opposite strands, which are DSBs by default. Alternatively, the MMR pathway functions in converting distal SSBs into DSBs (4).

Generation of DNA breaks

The BER pathway plays an important role during CSR. Among the BER proteins that are critical for removing the uracils generated by AID is the enzyme Ung. In Ung-deficient B-cells, S region DSBs are significantly decreased and consequently CSR is decreased by 95% (85, 258, 259). The abasic sites, generated by Ung-mediated removal of the uracil residues, is converted to SSBs by the purinic/apyrimidinic (APE1/APE2) endonucleases that have been shown to be important in CSR (89). The DNA breaks generated by Ung and APE endonucleases mostly occur at AID hotspots within the Sμ regions, indicating these proteins act at the cytosine residues that were targeted by AID (89, 259).

During the canonical BER pathway, the DNA nick generated by Ung is repaired by error-free DNA polymerases, such as Pol β, Pol δ, and Pol ε, and the DNA nick is sealed by DNA ligase 3 to complete the repair (95, 260, 261). Interestingly, Pol β is also functional during CSR and is involved in the faithful repair of S region DNA damages, thus, limiting the number of SSBs and reducing CSR (108). It has been suggested that during CSR, error-free DNA polymerases, such as Pol β, are functional, but, due to the great number of AID-induce DNA lesions, the error-free DNA polymerases are overwhelmed and DNA breaks remain to initiate the recombination machinery (108).

Conversion of SSBs to DSBs

As mentioned above, SSBs that are far from each other can be converted to DSBs needed for CSR. This function is attributed to the proteins involved in the MMR pathway. The MMR complex, therefore, makes CSR more efficient, although it is not essential and mice that lack MMR genes, *Msh2*$^{-/-}$, *Msh6*$^{-/-}$, *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$, or *Exo1*$^{-/-}$, display 2-7 fold decrease in CSR depending on the gene and the Ig isotype (96, 262-267).

The MMR pathway is initiated by recognition of U:G mismatches by the Msh2-Msh6 heterodimer (94). This is followed by recruitment of Mlh1-Pms2 heterodimer (268), which in human cells has endonuclease activity (269). Additional proteins are recruited to the MMR complex, such as the exonuclease Exo1, and a number of DNA replication factors such as PCNA and its loading

factor, replication factor C (RFC). Together, they function to excise the DNA segment containing the mutated nucleotide (Figure 9, (4, 270)). It is hypothesized that EXO1 excises the DNA strand past the mismatch until it reaches the SSB on the other DNA strand, thus creating a DSB (4).

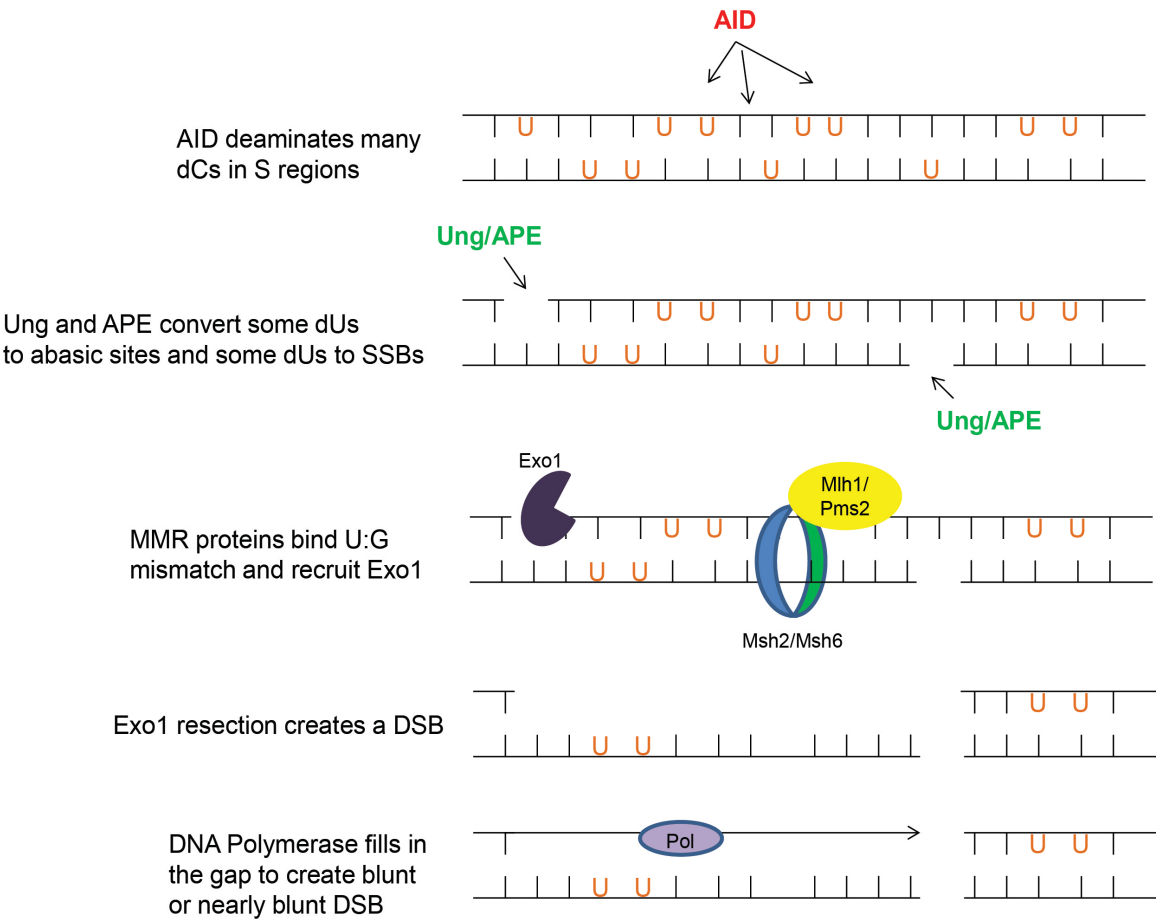

Figure adapted from Stavnezer J. *Trends in Immunology* 2011

Figure 9

Figure 9. Model for the generation of double-stranded DNA breaks.

Deamination of deoxycytidine (dC) to uracils (U) by AID generates single-stranded DNA breaks (SSBs) when the uracils are removed by Ung and APE. The SSBs are converted to double-stranded DNA breaks (DSB) when proteins involved in mismatch repair (MMR) pathway excise the DNA containing the mismatch.

Evidence for the role of MMR in converting SSBs into DSBs comes from mice that are double deficient in the MMR gene and the SμTR region. For example, CSR is only decreased by two-fold in SμTR knockouts, while *Msh2*$^{-/-}$:*SμTR*$^{-/-}$, *Mlh1*$^{-/-}$:*SμTR*$^{-/-}$, or *Exo1*$^{-/-}$:*SμTR*$^{-/-}$ double knockout mice display an almost complete block in CSR (217, 271, 272). Furthermore, switch junctions in *Msh2*$^{-/-}$ B-cells occur within the SμTR (262), and deficiencies in MMR proteins can result in decreased S region DSBs and in changes in the length of the switch microhomology junctions, suggesting that the MMR pathway is involved in processing DNA nicks (96, 263, 267, 272-274). Once DSBs are generated, the 5' or 3' ssDNA overhangs will be either excised or filled in by DNA polymerases to create the blunt DSBs needed for the non-homologous end joining (NHEJ) pathway that functions to join the S regions (4, 268, 275).

Non-homologous end joining

The NHEJ pathway is involved in the repair of DSBs generated by ionizing radiation, oxidative free radicals, V(D)J recombination, and CSR (4). Unlike the homologous recombination (HR) pathway, which is reportedly only active during S and G2 phase, NHEJ is active during non-replicative phase of the cell cycle (276-279). Because S region DSBs are generated and repaired in G1 (273), the NHEJ pathway plays an important role in CSR. Components of NHEJ that are important for CSR include the Ku70-Ku80 heterodimer, which is likely the first proteins to bind broken DNA ends, and DNA-ligase IV and Xrcc4 (280-283). Ku70 and Ku80 bind DNA ends at DSBs and mediate direct end-to-end joining of

the two DSBs (284). The kinase, DNA-PKcs then binds each end of the DSB, is phosphorylated, and phosphorylates the Ku70-Ku80 complex and Xrcc4 (285, 286). DNA-PKcs and another protein, Artemis, are involved in joining DNA ends that require processing (287). The ligation step involves Xrcc4 which aids DNA ligase IV in the ligation of dsDNA ends in S regions that are blunt, or have short compatible ends, generating very little microhomology (0-1 bp) in the DNA junctions between the Sμ and Sx region (4). Recently, it was shown that the S-S junctions in *Xrcc4*$^{-/-}$ B-cells, display increased junctional microhomology lengths (≥10 bp) (288), suggesting that besides the NHEJ pathway, alternative end-joining (A-EJ) pathways, that prefer to join S regions by microhomology mediated ligation, are also functional during CSR (288, 289).

Additional class switch recombination factors

The Mre11-Rad50-Nbs1 (MRN) complex can scan for and bind at DSBs (290, 291). This binding allows the ataxia telangiectasia mutated (ATM) kinase to bind Nbs1, activating ATM, which in turn phosphorylates several other proteins such as Nbs1, 53BPI, p53, Mdc1, and H2AX (292-295). ATM is, therefore, an important regulator of DNA damage repair and cell-cycle control (296, 297). CSR is reduced in B-cells that are missing components of the MRN complex (292, 298-300) and ATM (301, 302).

Among the proteins that have the most profound effect on CSR is 53 binding protein 1 (53BP1). CSR is reduced about 90% in *53bp1*$^{-/-}$ B-cells (303).

As the name implies, 53PB1 binds p53 and is a transcriptional coactivator of p53 (304, 305). 53BP1 is accumulated at DSB sites, and can induce the phosphorylation of ATM (306, 307). Recently, it was shown that 53BP1 is important in promoting long range CSR and promoting DSB repair through the NHEJ pathway (308, 309).

H2AX is a variant of histone H2A (310) that is important for CSR (311, 312). Very soon after the formation of DSBs, H2AX becomes phosphorylated (phosphorylated nomenclature is γH2AX) by ATM (313). γH2AX is estimated to spread over megabases (Mb) surrounding the DNA break (314), and serves as a docking site for other phosphorylated proteins to bind near the DSB (315).

Finally, the mediator of DNA damage checkpoint protein 1 (Mdc1) is also recruited to DSBs and phosphorylated by ATM (295, 316). Mdc1 is important to keep the MRN/ATM/γH2AX complex stable. CSR is only mildly reduced in *Mdc1*$^{-/-}$ B-cells (317).

Alternative end-joining pathways

Non-homologous end joining (NHEJ) and homologous recombination (HR) are the major DNA repair pathways which have been extensively studied (276, 277). Other DNA repair pathways, however, have also been identified and shown to play a role during CSR. One such pathway, appropriately called the alternative end-joining (A-EJ) pathway, was identified when the core NHEJ factors (Ku70,

Ku80, Xrcc4 and DNA ligase IV) were deleted (283, 288, 289). The A-EJ pathway is characterized by increased microhomology at switch junctions (1-20 nucleotides), and frequent aberrant chromosomal recombinations (translocations) (318). The components of the A-EJ pathway, however, are still mostly unknown. Studies in NHEJ-mutant yeast strains have identified a similar pathway, termed the microhomology-mediated end-joining (MMEJ) pathway, where key components have been identified, providing investigators with a number of candidate genes (Rad1, Mre11, Sae2, Tel1) to test during CSR (319-324). Recently it was shown that Msh2, Mlh1, and Exo1 proteins are important in generating the DSBs needed for both the A-EJ pathway and the NHEJ pathway during CSR (325). In addition, similar to the NHEJ pathway, the MRN complex has been suggested to be important for A-EJ (326-328).

Interchromosomal class switch recombination

Unlike V(D)J recombination when Ig alleles are asymmetrically located in the nucleus to ensure monoallelic expressing of one Ig allele, i.e. allelic exclusion (329, 330), in activated B-cells, S region GL transcription occurs at similar levels on both Igh alleles (331). Subsequently, CSR occurs on both alleles (332), and the S regions on the same chromosome are joined, i.e. intrachromosomal or *cis*-CSR takes place (333, 334). Although intrachromosomal CSR is the major mechanism of isotype switching, a significant level (7-14%) of interchromosomal, or *trans*-CSR, characterized by the recombination between Igh homologs, has been observed in mice designed to optimize the detection of interchromosomal

switching events between the paternal and the maternal Igh chromosomes (335, 336). More specifically, heterozygous mice that contain one non-functional μ^a allele, due to a mutation upstream of the Eμ enhancer which prevents the production of functional VDJ-Cμ protein, and one functional μ^b allele can produce up to 7% of IgA and 14% of IgG3 by trans-CSR (335). Earlier studies in rabbits have also shown that a significant level of IgA is produced by trans-CSR and not by trans-splicing or trans-gene conversion (337, 338).

The mechanism of interchromosomal CSR is not fully understood, however, the process must depend on AID because all CSR is abolished in AID-deficient B-cells (8). Recently it was shown that both intrachromosomal and interchromosomal CSR do not depend on Msh5 (339) and that *trans*-S-S joining is similar to *cis*-S-S joining in that both have similar junctional microhomology, and therefore, both probably are mediated by the NHEJ pathway (336, 339).

Interchromosomal CSR has also been observed between the endogenous Igh locus (chromosome 12) and Igh transgenes inserted on a chromosome other than chromosome 12 (176, 177, 181, 340). Interestingly, interchromosomal CSR between the endogenous Igh locus and an Igh transgene occurred in the absence of Igh 3'RR enhancers (181), which raises the question on whether the 3'RR enhancers have a role in preventing recombination between two chromosomal homologs. Certainly, interchromosomal recombination between two homologous S regions, i.e. Sμ to Sμ, does not lead to a switch in the Ab

class, and therefore, could be considered wasteful. These questions, and the role of AID in Igh/transgene recombinations, will be addressed in this thesis.

3. Aberrant class switch recombination can lead to translocations between Igh loci and proto-oncogenes.

For a translocation to occur, two heterologous chromosomal loci must be broken and joined together. The interchromosomal joining can either be reciprocal, i.e. an exchange occurs between two heterologous chromosomes, or non-reciprocal, which is characterized by deletions/duplications of chromosomal segments (341). The ongoing DNA damage and recombination during B-cell development make the Ig loci a hotspot for translocations. Therefore, it is not surprising that translocations are a common feature of hematopoietic malignancies (342). Indeed, translocations that join the c-myc gene to the Igh locus is a hallmark of Burkitt's lymphomas in humans (343).

3.1 Igh enhancers and c-myc translocations

Studies from mice, where the c-myc gene is joined to the Igh locus, have provided much insight into the molecular mechanisms of lymphomas. Upon translocation, the c-myc gene becomes associated with Igh enhancer elements which puts the c-myc gene under the transcriptional regulation of these enhancers, thereby, increasing c-myc expression and causing rapid proliferation

of B-cells (344). Transgenic mice that contain a transgene composed of the Igh Eμ enhancer, or the Igh 3'RR enhancers fused with the c-myc gene develop fatal lymphomas (345-347). Similarly, targeted insertion of Igh 3'RR enhancers upstream of the c-myc gene results in lymphomas (348). Studies from mice that are predisposed to develop c-myc/Igh associated B-cell lymphomas, and are also missing 3'RR enhancer elements HS3b and HS4, show that the 3'RR enhancers are not involved in promoting the translocation, but rather, just promote up-regulation of the translocated *c-myc* gene (349). Together these studies show that translocations that join proto-oncogenes with the Igh locus can cause lymphomas due to transcriptional activation of the proto-oncogenes by the Igh enhancer elements.

3.2 Aberrant class switch recombination

The CSR mechanism has been linked to some interchromosomal translocations that appear to be involved in oncogenesis (341, 342, 350). It has been suggested that these interchromosomal translocations reflect aberrant CSR activity acting at oncogene loci (such as c-myc) to cause recombination between the Ig S regions and the oncogene sequences (351). In fact, the frequency of such translocations increases in B-cells that are missing essential NHEJ factors, such as Ku80, Xrcc4, and DNA ligase 4 (352-354). These results indicate that the A-EJ pathway is more prone to cause translocation in B-cells when the NHEJ pathway is non-functional. Indeed, analyzing translocation junctions in NHEJ-

deficient B-cells, show an increase in microhomology mediated joining, further suggesting that the A-EJ pathway might be more prone to causing translocations (355). However, it is not clear why A-EJ would cause more translocation. One possibility, that still needs to be tested, is that without a functional NHEJ pathway, more DSBs would remain, therefore, leading to aberrant joining of chromosomes.

3.3 The role of activation-induced cytidine deaminase in translocations

Translocations have also been observed in B-cells lacking DNA damage response factors such as ATM, H2AX, 53BP1, and p53 (355). These proteins are involved in sensing DSBs and activating cellular responses to repair/cope with the damage (355), therefore, it is not surprising that deletions of any of these factors would result in genomic instability. In B-cells, AID causes significant DNA damage that could potentially be repaired in an aberrant manner causing translocations. A convincing number of studies, using IL-6 transgenic mice which are predisposed to developing B-cell lymphomas, have shown that translocations, such as c-myc/Igh joining, are dependent on AID (161, 356, 357). In fact, AID-induced DSBs have been detected in the c-myc region (358, 359). Contrary to these studies, AID-independent c-myc/Igh translocations, in mice immunized with the highly immunogenic substance, pristane, have been reported (360). The authors in this study argue that the translocation *per se* is not dependent on AID, but rather, the survival of the cells that have undergone a translocation (translocation positive cells) is dependent on AID. In support of this

idea, another group has shown that mice that overexpress the anti-apoptotic gene, *Bcl-xl*, on an AID-deficient background undergo c-myc/Igh translocations (361). However, even in this study, the frequency of translocations is higher in an AID-sufficient background. The role of AID in interchromosomal recombinations will be discussed further in this thesis.

4. Conclusion

B-cell diversification, such as SHM, CSR, and Ig V-gene conversion depends on the enzyme AID (7, 8). My work has concentrated on understanding the regulation of these Ig diversification pathways by using an Ig transgene.

Chapter 3 focuses on the role of AID in interchromosomal Igh/transgene translocations. My data show that Igh/transgene translocations are dependent on AID and that the recombination mechanism does not appear to involve trans-switching between the transgene Sμ region and the endogenous Sμ region. These results indicate that during trans-CSR, the recombination mechanism may be regulated such that the frequency of S regions joining with their homolog may be low in order to maximize B-cell responses to antigens.

Chapter 4 focuses on the role of AID in Ig V-gene conversion and SHM at the transgene locus. My data show that, although a transgene that is missing Igh

cis elements downstream of the Cμ gene, it is capable of accumulating AID-induced mutations, Ig V-gene conversion is not detected. Furthermore, mutagenesis at the transgene locus depends on Ung and Msh2 proteins. These results indicate that Igh *cis* elements 5' of the Cμ are sufficient to promote some level of mutagenic function of Ung and Msh2 and that the extent of DNA damage may dictate how the damage is resolved. I speculate that higher level of AID function and mutagenesis is needed for gene conversion.

Chapter 5 focuses on the role of p21 in SHM. My data show that B-cells express p21 after activation but the levels decrease at the time when AID is expected to be expressed in the cells. This regulation does not depend on AID since p21 mRNA levels are the same in both wildtype and $AID^{-/-}$ B-cells. SHM, however, is not affected by the absence of p21. These results indicate that regulation of p21 in activated B-cells is probably more important for maintaining proper cell cycle progression as opposed to promoting SHM of Ig genes.

CHAPTER 2

Materials and Methods

Mice

All experiments with mice were approved by and performed in accordance with the regulations of the Tufts University School of Medicine IACUC. The VV29 transgenic mice and AID knockout mice have been described elsewhere (8, 65, 66). The VV29 and AID$^{-/-}$ mice were crossed to generate VV29:AID$^{-/-}$ mice. AID knockout mice were obtained from Thereza Imanishi-Kari (Tufts University School of Medicine, Boston, Massachusetts) with permission from T. Honjo (Kyoto University, Kyoto, Japan). UNG-deficient mice (362) were obtained from Janet Stavnezer (University of Massachusetts Medical School, Worcester, Massachusetts) with permission from T. Lindahl and D. Barnes (London Research Institute, London, England). Msh2-deficient mice (363) were obtained from Janet Stavnezer with permission from T. Mak (University of Toronto, Toronto, Canada). The VV29 mice were bred to Ung/Msh2 double knockout mice to generate transgenic VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice. Mice deficient in p21 (*Cdkn1a*$^{-/-}$) have been described elsewhere (139), are phenotypically normal up to 7 months of age (364), and were obtained from Richard Van Etten (Tufts University School of Medicine, Boston, Massachusetts). All mice were maintained in a pathogen-free mouse facility at Tufts University School of Medicine.

Immunization

For *in vivo* translocation experiments and Ig V-gene conversion experiments: Mice received four intraperitoneal (i.p.) immunizations with p-azophenylarsonate (Ars) conjugated to keyhole limpet hemocyanin (KLH) as previously described (65, 68). For some mice (as indicated in the figures), only a primary immunization was administered by one i.p. injection and spleens were harvested for analysis one week later. For each genotype, a cohort of at least 5 mice were used for each immunization.

For somatic hypermutation experiments: Mice received one i.p. immunizations with Ars-KLH as previously described (65, 68). This immunization was repeated one more time three weeks later and spleens were harvested one week after the second immunization.

RNA isolation, cDNA synthesis, and PCR

Total RNA was isolated with TRIzol following the manufacture's protocol (Invitrogen). One microgram of RNA was used for cDNA synthesis using oligo$(dT)^{20}$ and SuperScript III as recommended by the manufacturer (Invitrogen). The cDNA was used for PCR amplification of Cγ transcripts using CγRI reverse primer, which hybridizes to the C_H1 exon of either Cγ1, Cγ2a, or Cγ2b (65, 67), and forward primer L3RI, which hybridizes to the Leader exon of both the VV29 transgene V-genes (67) and up to 10 endogenous V-genes (see semi-quantitative PCR below). For amplification of transgene-specific Cμ transcripts (VV29-Cμ), a transgene specific forward primer, TND (also used as a

probe, see Southern blots below) (68) and Cμ4R reverse primer (located on exon 4 of the Cμ gene, 5'TGGACTTGTCCACGGTCCTCT) were used. Amplification of endogenous Cμ transcripts was performed with a forward Cμ1F primer (located on exon 1 of the Cμ gene 5'GTCAGTCCTTCCCAAATG) and the Cμ4R primer. The PCR conditions for VV29-Cμ transcripts were 55°C annealing temperature for 30 seconds and 72°C extension temperature for 1.5 minutes for 35 cycles. For some samples, the RNA was DNase I treated prior to the cDNA synthesis as described by the manufacturer (Invitrogen). As loading controls, or for DNA contamination controls, RT-PCR amplification of β-actin was performed using β-actin forward (5'AGACTTCGAGCAGGAGATGG) and β-actin reverse (5'CACAGAGTACTTGCGCTCAG) primers at 55°C annealing temperature for 30 seconds and 72°C extension temperature for 1 minute for 35 cycles. The β-actin primers were designed to give a 300 base pair product from an mRNA transcript template and an 800 base pair product from genomic DNA templates that might be present due to DNA contamination.

Southern Blot assay

PCR products were separated by agarose gel electrophoresis and transferred onto Zeta-Probe nylon membranes (Bio-Rad). Oligonucleotide probes were end-labeled with [γ-32P]ATP (MP Biomedicals) using OptiKinase as described by the manufacturer (USB) and purified by NucAway Spin Columns (Ambion) before hybridization at 42°C in 3XSSC/0.1%SDS/10X Denhardt's Solution/50 μg/ml salmon sperm DNA (Roche) hybridization buffer. The following probes were

used: TND, located in the VDJ junctions of the VV29 transgene (68), endogenous Cμ probe, located in exon 1 of the C57BL/6 Cμ gene (5'GCAAAAACAAAGATCTGC), and the Transgene Cμ probe, located in exon 1 of the BALB/c Cμ gene (5'GCAAAAACAGAGATCTGC). All the blots were washed once in 3X SSC/5 mM EDTA/0.1% SDS/5X Denhardt's Solution/50 μg/ml salmon sperm DNA (Roche) and once in 1X SSC/0.1% SDS/5 mM EDTA for 15 minutes each at 42°C. For Cμ probes, the blots were further washed twice in 0.1X SSC/0.1% SDS/5 mM EDTA for 30 minutes each at 42°C.

Translocation assay

Cγ transcripts containing transgene VDJ segments or endogenous VDJ segments were PCR amplified from serially diluted cDNA (as indicated in Figure 2A) with primers L3RI and CγRI. The PCR annealing temperature was 55°C for 30 seconds and an extension temperature at 72°C for 1 minute for 40 cycles. The PCR products were transferred onto Zeta-probe nylon membranes (Bio-Rad) and hybridized with a transgene-specific probe (TND) to identify transgenic VV29-Cγ transcripts. Amplifications of β-actin with the β-actin primers listed above were used as loading controls. The β-actin PCR was performed with cDNAs that were diluted at 1:6400, 1:12800, and 1:25600. Quantitation was performed by measuring band intensities from Southern blots for transgene-specific Cγ transcripts (VV29-Cγ), or band intensities from ethidium bromide-stained agarose gels for β-actin, followed by dilution factor correction. The mean

values from three independent experiments were normalized by dividing the values for the VV29-Cγ to the values obtained for β-actin.

Translocation frequency

Cγ transcripts from *in vitro* stimulated B-cell cultures using L3RI and the CγRI primers were amplified using Platinum *Taq* DNA Polymerase (Invitrogen). The PCR products were cloned into pGEM vectors (Promega) and plasmids containing the PCR inserts were isolated as described previously (365). Forty plasmids were spotted onto a Zeta-probe nylon membrane for dot blot hybridization with the TND probe using the method described above. All clones (both TND-positive and TND-negative) were sequenced at the Tufts University Core Facility (Tufts University School of Medicine, Boston, MA). The sequence analyses confirmed the association of transgene VDJ sequences with endogenous Cγ sequences for TND-positive clones and provided a frequency of 27.5% (11 TND-positive clones/40 total clones) for translocation products among the PCR clones. Among the TND-positive clones only one nucleotide difference was noted in comparison to the transgene VDJ sequence, indicating a low PCR error rate. Next we analyzed the sequences of the 29 TND-negative clones to estimate the number of possible V-genes that can be amplified with the V-gene primer, L3RI. We determined that at least 10 V-genes, or 9% of the functional V-genes (assuming that all of the functional 110 V-genes that are available (14) are expressed), can be amplified with the L3RI primer. This analysis provides an approach to estimating the percentage of switch events in the stimulated B cells

that lead to chromosomal translocations. This approach relies on assumptions that are outlined in the Discussion. The frequency of translocations is considered to be indicated by (total number of translocations)/(total number of switch events) in the stimulated population. We calculate the total number of switch events as (100-27.5)(10/110)+27.5 = 825 (in arbitrary units) and then the translocation frequency as 27.5/825= 0.033 or 3.3%.

Two-Color Fluorescence In Situ Hybridization

Two-color FISH was used to label the 3' region of the Igh locus using BAC199 (a gift from Fred Alt at Harvard Medical School, Boston, Massachusetts with permission from Barbara Birshtein, Albert Einstein College of Medicine, New York), which encompasses the Igh 3' enhancer and 100 kb downstream (366), and the Cμ gene using an 8 kb plasmid containing the VV29 R16.7 VDJ segment, the Igh intronic Eμ enhancer, and the Cμ gene (referred as the Cμ probe throughout this paper). BAC199 was labeled with biotin and the 8 kb Cμ plasmid was labeled with digoxigenin by nick translation (Roche) as per manufacturer's instructions and as described previously (366). Metaphases were prepared from VV29 or C57BL/6 splenic B-cells stimulated for 24 hours with 25 μg/ml lipopolysaccharide (LPS) (Sigma) and 10 ng/ml interleukin-4 (IL-4) (PeproTech). Stimulated B-cells were then frozen in metaphase by incubating with colcemid (KaryoMax, Invitrogen), then swollen in KCl and fixed in 3:1 methanol/acetic acid as previously described (366). Metaphase images were captured using Olympus BX50 microscope with Isis v5.1.2 software

(MetaSystems) at the Cytogenetics Laboratory at Tufts University Medical Center. Thirty-five metaphases were analyzed for VV29 transgenic strains and 15 metaphases were analyzed for C57BL/6 strains.

B-cell isolation

Splenic B-cells were isolated by negative selection using B-cell isolation kits (Stemcell Tech). Two million B-cells were stimulated at a concentration of $0.5x10^6$ cells/ml. RPMI-1640 (BioWhittaker) media, supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 1X L-glutamine, 1X non-essential amino acids, 1X penicillin:streptomycin, and 1X sodium pyruvate (all from Invitrogen) was used.

Class switch recombination

B cells were isolated as described above. Two million B cells were activated for four days to induce CSR to IgG1 (25 μg/ml LPS + 20 ng/ml IL-4), IgG3 (25 μg/ml), IgG2a (25 μg/ml LPS + 100 ng/ml IFN-γ), IgG2b (25 μg/ml LPS + 0.5 ng/ml TGF-β) as described elsewhere (272).

Flow cytometry

Four days post stimulation, B cells were stained with Alexa Fluor®467-conjugated anti-B220 (Invitrogen), and either with Alexa Fluor®488-conjugated anit-IgG1, anti-IgG3, anti-IgG2a, or anti-IgG2b (Invitrogen). Propidium iodide (PI) was added just prior to FACS analysis with FACSCalibur (BD Biosciences).

Cell Sorting

B-cells were stained with IgM (IgM-PE, Southern Biotech) or the B-cell marker, B220 (CD45R/B200-PE, Southern Biotech), and the B-cell activation marker, peanut agglutinin (PNA) (PNA-FITC, Vector Laboratories Inc.) by standard methods. Activated IgM+ B-cells were isolate by sorting for $IgM^{+}PNA^{high}$ or $IgM^{high}PNA^{high}$ populations and activated B-cells were isolated by sorting for $B220^{+}PNA^{high}$ populations as described elsewhere. The MoFlo instrument (Dako Cytomation) was used for sorting cells.

Quantitative Real-time PCR

B cells were isolated from splenoctyes and two million B cells were stimulated for 8hr, 24hr, 48hr, 72hr, and 96hr with LPS and IL-4 as described above. Unstimulated cells (time 0hr) were lysed in TRIzol (Invitrogen) after B cell isolation. Total RNA was isolated and treated with DNase I prior to cDNA synthesis as described above. Real-time PCR was performed with SYBR® Green PCR master mix (Applied Biosystems) with mouse p21 primers (forward- 5' CACAGGCACCATGTCCAATC, and reverse- 5' GACAACGGCACACTTTGCTC) and mouse β-actin primers (for endogenous control) (forward- 5' AGGTATCCTGACCCTGAAC, and reverse- 5' CACACGCAGCTCATTGTAG) in a IQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad). WEHI-231 murine B-cell lines were used to make standard curves for p21 and β-actin expression. Relative quantification was determined from the standard curves and by normalizing to β-actin. Real-Time PCR data

represent the means that were obtained from triplicates from three independent experiments. Student's *t*-tests were performed to determine statistical differences.

Semi-Quantitative PCR

Splenocytes of immunized mice were harvested and RNA isolation/RT-PCR was performed as described above. VDJ-Cμ and VDJ-Cγ transcripts were PCR amplified from serially diluted cDNA (as indicated in the Supplementary figures) with L3RI forward primer (see above) and Cμ reverse primer (see above) or CγRI reverse primer (see above). PCR products where transferred onto Zeta-probe nylon membranes (BioRad) and hybridized to the transgene-specific probe as described above.

Somatic hypermutation

For p21 experiments: Activated B cells were sorted from Peyer's patches of 2-4 month old *Cdkn1a*$^{-/-}$ (p21$^{-/-}$) mice (4 mice per experiment, a total of three independent experiments), *Cdkn1a*$^{+/+}$ (p21$^{+/+}$) littermate controls (4 mice per experiment, a total of 2 independent experiments) and *Cdkn1a*$^{+/-}$ (p21$^{+/-}$) littermate controls (4 mice in one experiment). Activated B cells (B220^{+}PNA^{high}) were sorted and lysed for DNA isolation using Aqua Pure Genomic DNA isolation kits (Bio-Rad). The Jh2 to Jh4 intronic region (~1.4 kilobases) of the Igh locus was amplified using the Jh2-forward primer; 5' GGCACCACTCTCACAGTCTCCTCAGG and Jh4-reverse primer; 5'

TGAGACCGAGGCTAGATGCC. High fidelity *Pfx Platinum Taq DNA Polymerase* was used for the amplification process as described by the manufacturer (Invitrogen). The PCR error rate was $2.2x10^{-4}$ which was determined from sorted naïve B cells ($B220^{+}PNA^{low/-}$). The PCR products were separated on an agarose gel and the 1.4 kb products were isolated from the gel using QIAquick Gel Extraction kits (Qiagen). Gel purified PCR products were cloned into pGEM vectors (Promega) and transformed into 5-alpha F'I^{q}competent *Escherichia coli* (New England BioLabs). Plasmids containing individual PCR clones were isolated as described before (365). Each clone was sequenced bidirectionally with two different primers (T7 and Sp6) that hybridize to the pGEM vector (Promega). Sequencing was performed at the Tufts University Core Facility (Tufts University School of Medicine, Boston, MA). The sequences were analyzed with Clone Manager software (Sci-ED) and SHM was determined by dividing the total number of mutations that were observed by the total number of bp that were analyzed. Clones that contained identical mutations, and clones that shared some mutations, were counted once. In my analysis, only 2 clones shared mutations, one clone had 15 mutations that were identical to the sister clone which had 5 additional mutations; of these, only the clone with the most mutations was included in the analysis. Among the identical clones, 5 sets of clones (10 total clones) contained identical mutations and each set was counted as one clone. The sequence specificity data only includes clones that were counted in the pie charts. Both the mutation frequency and the mutation patterns were confirmed by the SHMTool webserver (http://scb.aecom.yu.edu/cgi-bin/p1)

(367) and corrected for base composition. *p* values were calculated using two-tailed unpaired Student's *t*-tests.

For VV29 experiments: Activated B-cells ($B220^{+}PNA^{high}$) were harvested from immunized mice and total RNA was isolated and RT-PCR was performed as described above. The cDNA was PCR amplified with the forward primer L3RI (see above) and a reverse primer, Cμ4R (see above). The sequences were analyzed to distinguish transgene VDJ regions from endogenous VDJ regions and whether the VDJ regions are associated with transgene Cμ gene or the endogenous Cμ gene. Transgene Cμ gene was distinguished from the endogenous Cμ gene due to allotypic differences between the two genes (see above). SHM was determined by comparing the 301 bp transgenic V-regions to the original VV29 V-region sequence, and by comparing the 301 bp endogenous V-regions to germline V-gene sequences. NCBI Blast was used to identify germline V-genes. Clone Manager software (Sci-ED) was used to analyze the sequences. SHM was determined by dividing the total number of mutations that were observed by the total number of bp that were analyzed. Clones that contained identical mutations, and clones that shared some mutations, were counted once.

Immunoglobulin V-gene conversion

Total RNA was isolated from the spleens, blood, and activated IgM+ B-cells of immunized mice as described elsewhere above. The cDNA was PCR amplified for Cμ, Cγ1, and Cγ3 (see reference (8) for primer sequences) transcripts using

the forward primer L3RI (see above). PCR products were separated by agarose gel electrophoresis and transferred onto Zeta-Probe nylon membranes (BioRad) for Southern blot analysis as described above. The following probes were used: TND, located in the VDJ junctions of the VV29 transgene and used to identify all transgenic VDJ regions; 2B4, located in the CDR2 region of the 2B4 VDJ segment and used to identify transgenic VDJ regions that have undergone Ig V-gene conversion events; and R16.7, located in the CDR2 region of the R16.7 VDJ segment and used to identify transgenic VDJ regions that have not undergone Ig V-gene conversion events (68). To confirm Ig V-gene, PCR products were cloned and hybridized with TND or 2B4 probes as described above. The clones that hybridized to the 2B4 probe (Ig V-gene conversion positive clones) were sequenced as described above.

CHAPTER 3

Translocation of an antibody transgene requires AID and occurs by interchromosomal switching to all switch regions except the mu switch region.

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Additional data not presented in the published manuscript are included in this chapter as *Appendix figures*.

For this chapter, Jennifer Eccleston did the *Fluorescence In situ Hybridization* experiment.

1. Introduction

Immunoglobulin (Ig) class switch recombination (CSR) occurs most often by intrachromosomal recombinations between switch (S) regions located on a single chromosome, but can also occur by interchromosomal recombinations between Ig heavy chain (Igh) S regions located on chromosomal homologs (4, 333, 334).

The CSR mechanism has been linked to some interchromosomal translocations that appear to be involved in oncogenesis (341, 342, 350). It has been suggested that these interchromosomal translocations reflect aberrant CSR activity acting at oncogene loci (such as c-myc) to cause recombination between the Ig S region and oncogene sequences (351). Interchromosomal translocations have also been observed for some transgenes in which transgene V-region sequences are translocated into the endogenous Ig locus using a process that appears similar to CSR (177, 368). However, the relationships of CSR between Igh-bearing chromosomal homologs to the recombinations between non-homologs that occur during oncogene/Igh and transgene/Igh translocations are not clear. In particular, several studies have differed regarding the AID-dependence of oncogene/Igh translocations (161, 356, 357, 359-361, 369, 370). In addition, no studies have yet tested the AID-dependence of transgene/Igh switching.

I have now investigated the role of AID in interchromosomal Ig transgene isotype switching by crossing AID deficient mice with transgenic mice (VV29) that exhibit transgene translocations (66).

2. Results

2.1 The VV29 transgene is not located on the same chromosome that carries the Igh locus.

To analyze the role of AID in transgene/Igh translocations, I have used the transgenic mouse, VV29, that carries two copies of a transgene that encodes two closely spaced anti-azophenlarsonate (anti-Ars) specific VDJ segments, the Eμ intronic enhancer, a 600 base pair Sμ tandem repeat region, and a Cμ gene segment (Figure 1A) and are very similar to previous higher copy transgenic mice that have been shown to exhibit transgene isotype switching by an interchromosomal translocation process (177, 368). I first determined whether isotype switching events in the VV29 mice represent interchromosomal translocation by performing fluorescence *in situ* hybridization (FISH) to show that the transgene is not inserted on the same chromosome that carries the Igh locus (chromosome 12). In Figure 1B-C, splenic B-cells from VV29 and C57BL/6 mice were stimulated with LPS and IL-4 for 24 hours and fixed in metaphase before hybridization with an 8 kb Cμ probe and a 100 kb Igh locus-specific probe encompassing the 3' Igh enhancer. The Cμ probe is specific for the Cμ gene region that is present in both the VV29 transgene and the endogenous Igh locus. As shown in Figure 1B, there are six Cμ signals (green) in the VV29 metaphase spreads. Four of these signals represent the endogenous Igh loci as shown by co-localization with the red Igh locus-specific signals that represent the sister

chromatids of two Igh alleles on chromosome 12. The two remaining Cμ probe signals are on sister chromatids of a chromosome different from chromosome 12 as indicated by the lack of hybridization with the Igh locus-specific probe. This experiment was repeated with a C57BL/6 mouse as a control to show the specificity of the Cμ probe and the Igh locus-specific probe. As shown in Figure 1C, C57BL/6 metaphase spreads only show four Cμ signals that co-localize with four red Igh signals. Based on these results I conclude that the integrated transgene in VV29 mice is not located on chromosome 12.

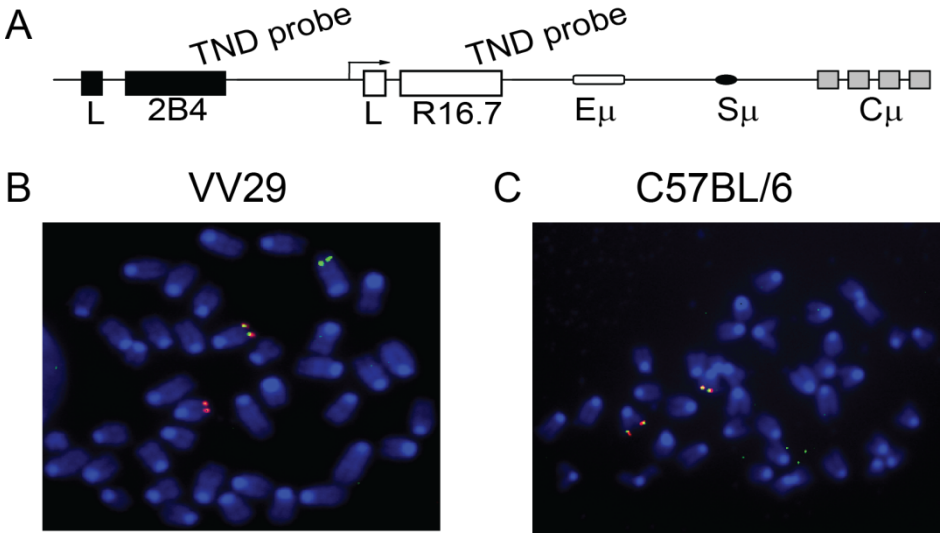


Figure 1

Figure 1. The VV29 transgene is not located on the same chromosome that carries the Igh locus. (A) The VV29 transgene consists of two closely spaced tandem VDJ segments (2B4 and R16.7), the Eμ intronic enhancer, a 600 base pair Sμ switch region, and a Cμ H-chain. The two VDJ segments respond to the Ars hapten (*p*-azophenylarsonate). The first VDJ (2B4) is promoterless while the second VDJ (R16.7), situated 1.5kb downstream, has a promoter (black arrow). The location of the TND probe, which spans the VDJ junction, is depicted on the diagram. L: Leader exon. Diagram not drawn to scale. **(B-C)** Two-color fluorescent *in situ* hybridization of B-cells isolated from (B) VV29 transgenic and (C) C57BL/6 mice and stimulated for 24 hours *in vitro* with LPS and IL-4. Stimulated B-cells were fixed in metaphase and hybridized to a H-chain specific probe (red), which encompasses the Igh 3'RR enhancer and 100 kb downstream, and Cμ probe (green). All chromatids where stained with DAPI (blue). At least 35 metaphases were analyzed for VV29 transgenic strains and 15 metaphases were analyzed for C57BL/6 strains.

2.2 AID is required for interchromosomal isotype switching.

To determine whether interchromosomal transgene isotype switching is dependent on AID, I crossed VV29 transgenic mice to AID deficient mice to establish AID deficient VV29 mice (VV29:AID$^{-/-}$). These mice, along with VV29:AID$^{+/+}$, VV29:AID$^{+/-}$, and nontransgenic C57BL/6 and AID$^{-/-}$ mice, were immunized with Ars-KLH and splenocyte RNAs were harvested for RT-PCR to assess the levels of transgene VDJ segments found to be associated with endogenous Cγ transcripts. The relative expression of transgene-derived Cγ transcripts (VV29-Cγ) was determined by semi-quantitative PCR followed by Southern blot hybridization using a probe (TND) specific for the transgene VDJ region. The results in Figure 2A show that VV29:AID$^{-/-}$ mice exhibit almost complete elimination of transgene-derived Cγ expression. The lack of hybridization of the TND probe to non-transgenic C57BL/6 Cγ PCR products verifies that the RT-PCR/Southern blot assay identifies only Cγ transcripts that are associated with VV29 VDJ segments. Based on the differences in the Southern blot band intensities for VV29-Cγ transcripts among the different mice strains, I am estimating that there is a 1,000 to 10,000-fold increase in the abundance of transgene-derived IgG mRNAs in VV29:AID$^{+/+}$ mice, indicating that AID plays a major role in interchromosomal isotype switching. The extremely low levels of transgenic IgG RNAs in a few VV29:AID$^{-/-}$ mice (3 out of 7 VV29/AID$^{-/-}$, data not shown) are possibly due to Ig DNA breaks that have resulted from an AID-independent mechanism, suggesting that it is possible for Ig DNA breaks to

rarely occur in the absence of AID. The dramatic increase in frequency of such events when AID is present indicates that the most prevalent mechanism for interchromosomal transgene isotype switching events is AID dependent.

I also wanted to determine whether AID-dependent interchromosomal isotype switching in VV29 mice is a frequent event or a rare event which is amplified by selection during immunization. In order to investigate whether interchromosomal events can occur in the absence of antigen selection, I stimulated VV29 B-cells with LPS and IL-4 and cultured them for four days to undergo CSR. Using the same PCR/Southern blot analysis as described above, I detected AID-dependent interchromosomal isotype switching events *in vitro* (Figure 2B, *Appendix Figure 1*). These translocations were not detected in VV29:AID$^{-/-}$ or nontransgenic AID$^{-/-}$ B-cells. I detected *in vitro* translocation events in about 3% of the Cγ transcripts as indicated by the number of cloned Cγ products that hybridize to the transgene-specific TND probe and by determining the number of V-genes that the forward V-gene primer (L3RI) can amplify. I identified 8 endogenous V-genes that are amplified and that have 100% homology to the forward V-gene primer, while 2 other endogenous V-genes were also amplified but are 96% and 81% homologous to the forward V-gene primer. This indicates that the primers are not biased to the transgene VDJ regions but can also efficiently amplify at least 9% of the V-genes if I assume that all 110 functional endogenous V-gene are expressed (see materials and methods, *Appendix Figure 2A-B*). Furthermore, based on previous publications (371), I

assume that transgene-induced allelic exclusion does not bias against intrachromosomal switching (see discussion). Taken together, my results indicate that AID is required for almost all interchromosomal translocations involving the VV29 transgene and the Igh locus, and that such AID mediated interchromosomal translocations occur at a relatively high frequency.

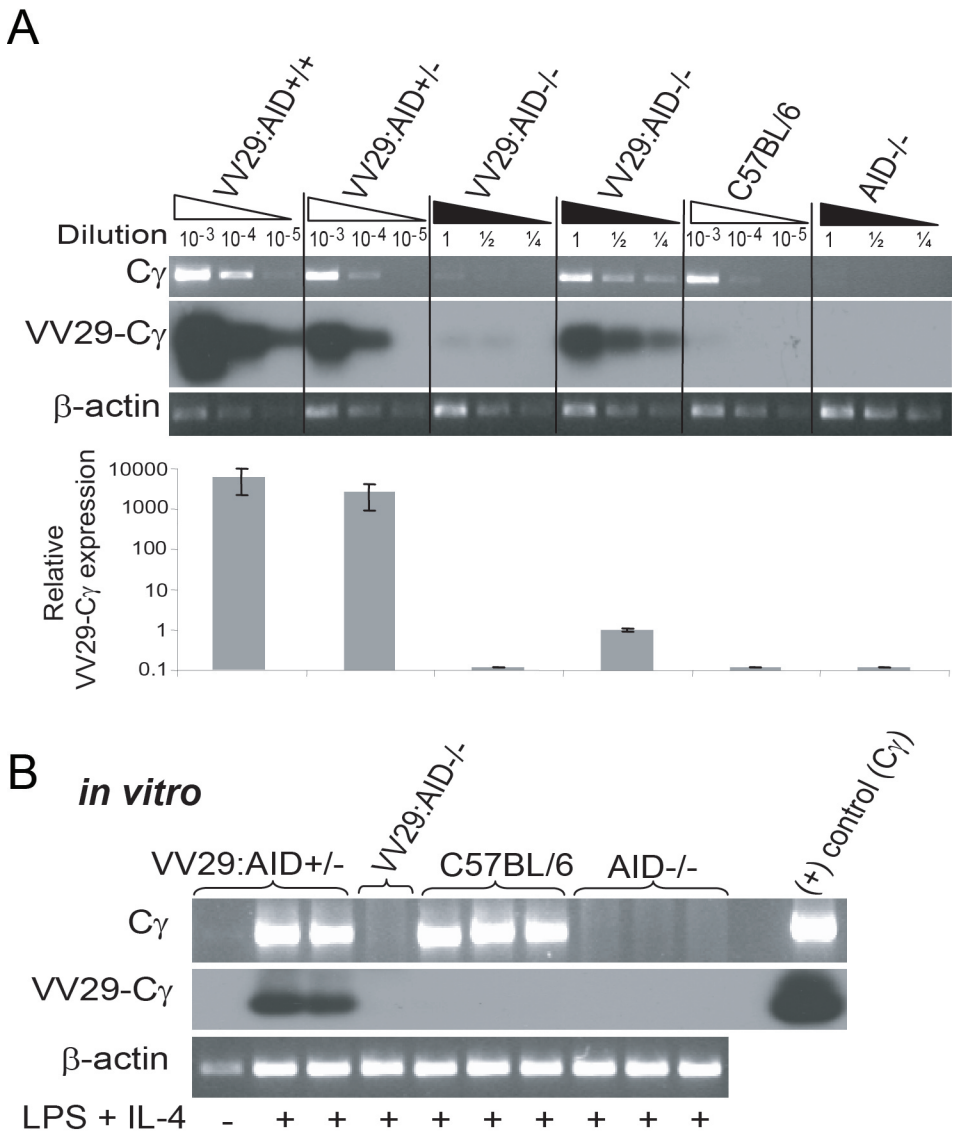


Figure 2

Figure 2. AID is required for interchromosomal isotype switching. (A) Upper panels: Semi-quantitative PCR/Southern blot assays of the indicated mice immunized with Ars-KLH. Splenocytes were harvested for cDNA synthesis. The cDNAs were diluted as indicated and Cγ transcripts were amplified by PCR. PCR products were subjected to Southern blot analysis and hybridized to a transgene-specific probe (TND) to identify Cγ transcripts that are associated with VV29 VDJ segments (VV29-Cγ). As a loading control and for normalization, β-actin was amplified from cDNA that was diluted at 1:6400, 1:12800, and 1:25600. Each set of three lanes represent data from a single mouse; sets 3 and 4 are from two different VV29:AID$^{-/-}$ mice. Lower panel: The band intensities of the Southern blots were quantified, normalized to β-actin (see materials and methods) and the data presented using a $\log_{10}$ scale. Each bar represents the mean values from three independent repetitions from a single mouse. Error bars represent standard deviation (SD) from three independent repetitions of the experiments using the same mice. **(B)** The translocation frequency was determined by stimulating B-cells isolated from the indicated mice with LPS and IL-4 for four days before RNA isolation and RT-PCR for Cγ transcripts. PCR products were subjected to Southern blot analysis and hybridized to a transgene-specific probe (TND) to identify VV29-Cγ transcripts. Amplification of β-actin serves as loading control. Each lane represents data from a single mouse.

2.3 Sμ to Sμ recombination is not found among interchromosomal switch events.

Due to the relatively high rate of transgene translocations into the endogenous Cγ region, I wanted to assess the pathway of the translocation process. Specifically, I wanted to determine whether translocation of the transgene could involve interchromosomal recombination with the endogenous Sμ region. I assayed for Sμ to Sμ recombination by determining whether, among B-cells stimulated to switch, transgene VV29 VDJ segments could be found associated with the endogenous Cμ gene rather than the transgene Cμ gene.

I was able to distinguish the endogenous Cμ gene from the transgene Cμ gene due to allotypic differences between these genes; the VV29 transgene contains the μ^a allele from BALB/c mice and the endogenous Cμ is derived from the C57BL/6 μ^b allele. Transgene specific primers were used to amplify VV29-Cμ transcripts, followed by Southern blot assays using oligonucleotide probes to distinguish the Cμ gene allotypes. Both *in vitro* (LPS + IL-4 stimulated B-cell cultures) and *in vivo* (immunization with Ars-KLH) results show that VV29 VDJ segments are found associated with the VV29 Cμ gene but not with the endogenous Cμ gene. In Figure 3A, VV29-Cμ transcripts, isolated from spleens of immunized mice, strongly hybridize to the transgene Cμ probe but not to the endogenous Cμ probe. Furthermore, VV29:$AID^{+/+}$ B-cell populations stimulated in culture with LPS and IL-4, in which all cells are activated, and a high frequency

of cells are undergoing CSR, also express VV29-Cμ transcripts that only hybridize to the transgene Cμ probe (Figure 3B). These findings indicate that interchromosomal switching events between the VV29 Sμ region and the endogenous Sμ region do not appear to mediate the translocation of the VV29 transgene into the Igh locus.

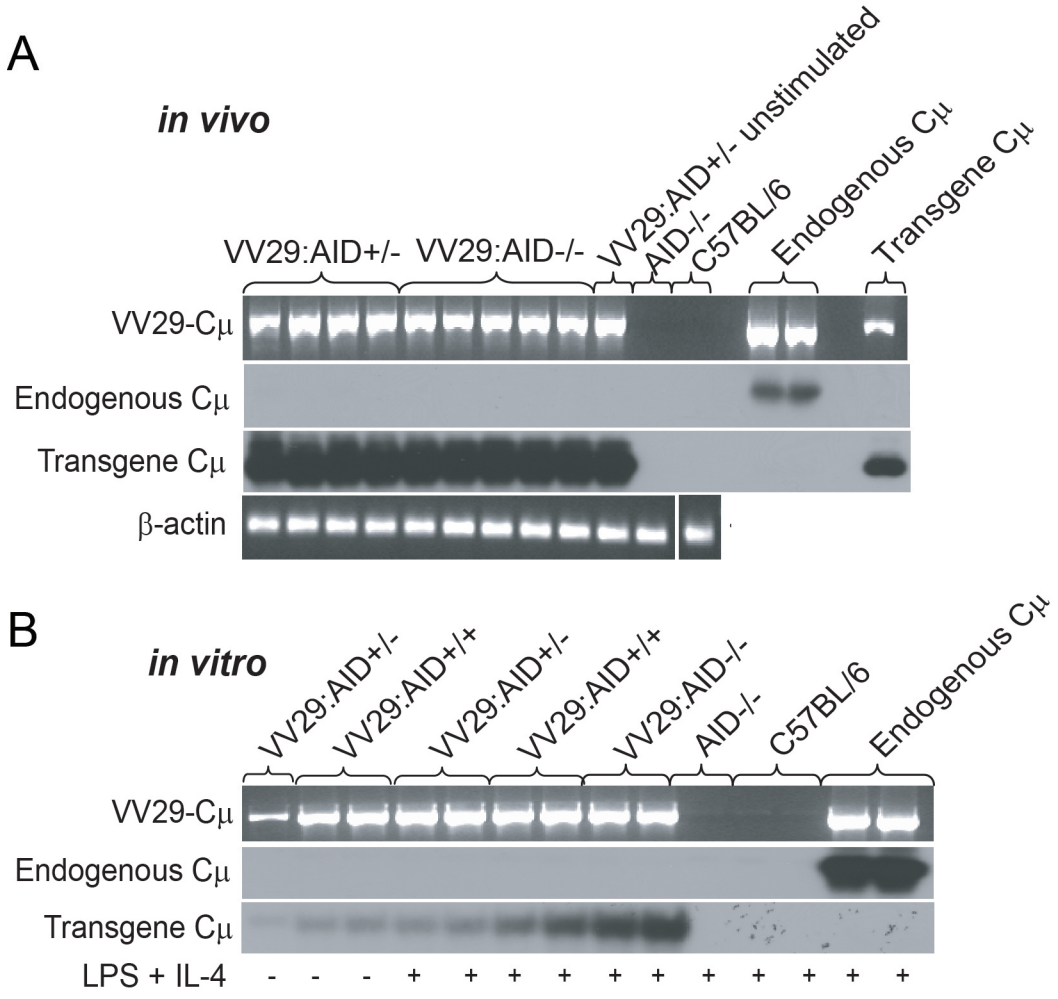


Figure 3

Figure 3. Sμ to Sμ recombination is not found among interchromosomal switch events. (A) The indicated mice were immunized with Ars-KLH and RNA was isolated from splenocytes for amplification of VV29-Cμ transcripts using the transgene-specific forward primer (TND) and the Cμ4R primer. Immunized C57BL/6 and non-transgenic $AID^{-/-}$ mice were used as negative controls to show the specificity of the transgene-specific TND primer. Endogenous Cμ transcripts were amplified from immunized C57BL/6 splenocytes using forward Cμ1F and reverse Cμ4R primers. Transgene Cμ is from a plasmid that contained the VV29 transgene Cμ gene which was confirmed by sequencing analysis. The PCR products where transferred onto two different membranes and Southern blots were performed with either a transgene-specific Cμ probe (Transgene Cμ) or an endogenous Cμ probe (Endogenous Cμ). β-actin PCR serves as loading control, white line indicates that the β-actin PCR for C57BL/6 was cut out from a different gel. Each lane represents data from a single mouse. **(B)** VV29 B-cells were stimulated with LPS+IL-4 for four days and RNA was isolated. The same RT-PCR/Southern blot assay as described in (A) was performed. Each lane represents data from a single mouse.

3. Discussion

Using a transgenic mouse model, I now show that transgene/Igh translocations that lead to isotype switching occur by AID-mediated recombination. Although transgene/Igh translocations occur frequently to Igh Sγ regions, I cannot detect analogous translocations between the transgene and endogenous Igh Sμ regions, indicating that Sμ switch regions may have evolved to prevent trans-switching, perhaps to avoid non-effective switching between Sμ regions on the two Igh homologs.

Interchromosomal switch recombination events between the VV29 transgene and endogenous Igh locus produce Cγ transcripts that are associated with VV29 VDJ segments. I find that these trans-switching events are AID dependent as VV29:AID$^{-/-}$ mice either do not produce these transgene-derived Cγ transcripts or they produce them at extremely low levels. Because very low levels of transgene-derived Cγ mRNAs have been observed in some VV29:AID$^{-/-}$ mice, a rare AID-independent mechanism for the generation of these Cγ transcripts does exist. Chromosomal translocations in general are dependent on DNA breaks; it seems possible that certain stimuli could cause DNA damage and breaks in an AID-independent manner that lead to these very low levels of transgene switching. For example, immunization with highly immunogenic reagents could cause cellular stress (372-374) that may lead to AID-independent Ig DNA damage. Supporting this notion, it has been reported that immunization

of mice with pristane can result in c-myc/Igh translocations in AID knockout mice (360, 361, 369). The low levels of transgene isotype switching observed in some, but not all, immunized VV29:AID$^{-/-}$ mice indicate that these AID-independent translocations are rare. Furthermore, VV29-Cγ transcripts were not produced in any VV29:AID$^{-/-}$ mice that received one dose of primary immunization (data not shown) or in any VV29:AID$^{-/-}$ *in vitro* stimulated B-cells, further supporting my conclusion that the high levels of interchromosomal switch events observed in VV29 mice are dependent on AID. These results are similar to a number of recent studies that clearly demonstrate an important role for AID in Igh chromosomal translocations that involve the c-myc gene (161, 356, 357, 359, 370), although the frequency of translocations induced by B-cell stimulation in VV29 mice appears to be much greater. I detected *in vitro* translocation events in about 3% of the Cγ transcripts (see Materials and Methods for the calculations leading to this result). This frequency was based on sequencing of all the PCR-amplified Cγ transcripts to determine the number associated with endogenous VDJ regions. Based on the published sequences for the 10 endogenous V-genes found among the PCR-amplified Cγ transcripts, the leader primer is 100% homologous to 8 of the endogenous V-genes, while the homologies of the primer to the 2 remaining endogenous V-genes are 96% and 81%. This indicates that the RT-PCR amplification of the transgene and endogenous V-gene Cγ mRNA's are likely to have similar efficiencies because the only differences in most of the amplifications are the sequences that intervene between the two primer sites. These intervening sequences are all H-

chain V-region sequences and are unlikely to cause a strong bias in the PCR amplification, therefore, I have assumed that the relative number of clones represents the relative number of recombined genes in the stimulated B-cell population. In addition, I have assumed that transgene-induced allelic exclusion does not bias against intrachromosomal switching. Previously reported studies of ARS5 mice, which are quite similar to VV29 mice but have much higher transgene copy number, have shown that about 25% of B-cells expressed the transgene μ^a allotype, while 75% of the B-cells either expressed endogenous μ^b allotype or both μ^b and μ^a allotypes (25% μ^b, 50% both μ^b and μ^a) (371). Furthermore, in ARS5 B-cells, reduction in transgene copy number correlated with reduced transgene μ^a expression (371) suggesting that even more inefficient allelic exclusion would be likely in lower copy mice like VV29. It should be noted, however, that it is possible that allelic exclusion in the VV29 mice is not similar to previous published similar strains and that I may be overestimating the translocation frequency in this study. Nevertheless, even if I overestimate the translocation frequency by a couple of orders of magnitude, the translocation frequency is still at least five orders of magnitude higher than the $2x10^{-8}$ *in vitro* translocation frequency observed between the Igh/c-myc loci (161). In addition, my calculations may underestimate the translocation frequency because it is unlikely that all of the 110 endogenous V-genes are expressed.

The higher translocation frequency in the VV29 mouse could be due to the presence of certain Ig *cis* elements which may increase targeting of the CSR

machinery to the VV29 transgene. For example, assembly of protein complexes that promote long-range CSR may be recruited more easily to the VV29 transgene due to the presence of the Sμ regions or the intronic Eμ enhancer. The Sμ region and the Eμ enhancer, however, may not be the only *cis* elements required to recruit recombination factors to the transgene. Indeed, previous studies have shown that transgenes lacking the Sμ region or Eμ enhancer can also undergo recombination with the endogenous Igh loci (340). Alternatively, it is possible that the lack of certain *cis* elements, such as the 3'RR enhancers located 28 kb downstream of the Cα gene, may promote increased interchromosomal translocation in VV29 mice. A recent report has shown that interchromosomal translocations between an Igh transgene and the endogenous Igh locus can be detected if the transgene (designated as Δ3'RR) is lacking Igh 3'RR enhancer regions, specifically the DNase I hypersensitive sites HS3a, HS1,2, HS3b, and HS4 (181). Based on this finding, the authors hypothesize that interaction between the 3'RR enhancer and the intronic Eμ enhancer may function as a protective mechanism against translocations. This hypothesis suggests the lack of the 3'RR on one chromosome could make trans-association of enhancers on different chromosomes more favorable. My data is consistent with this hypothesis and I have shown that these types of interchromosomal translocations reflect interchromosomal CSR based on the findings that AID activity is required. It should be noted, however, that in the VV29 transgenic mice, interchromosomal translocations can occur *in vitro*, whereas in Δ3'RR transgenic mice interchromosomal translocations can only be detected *in vivo*.

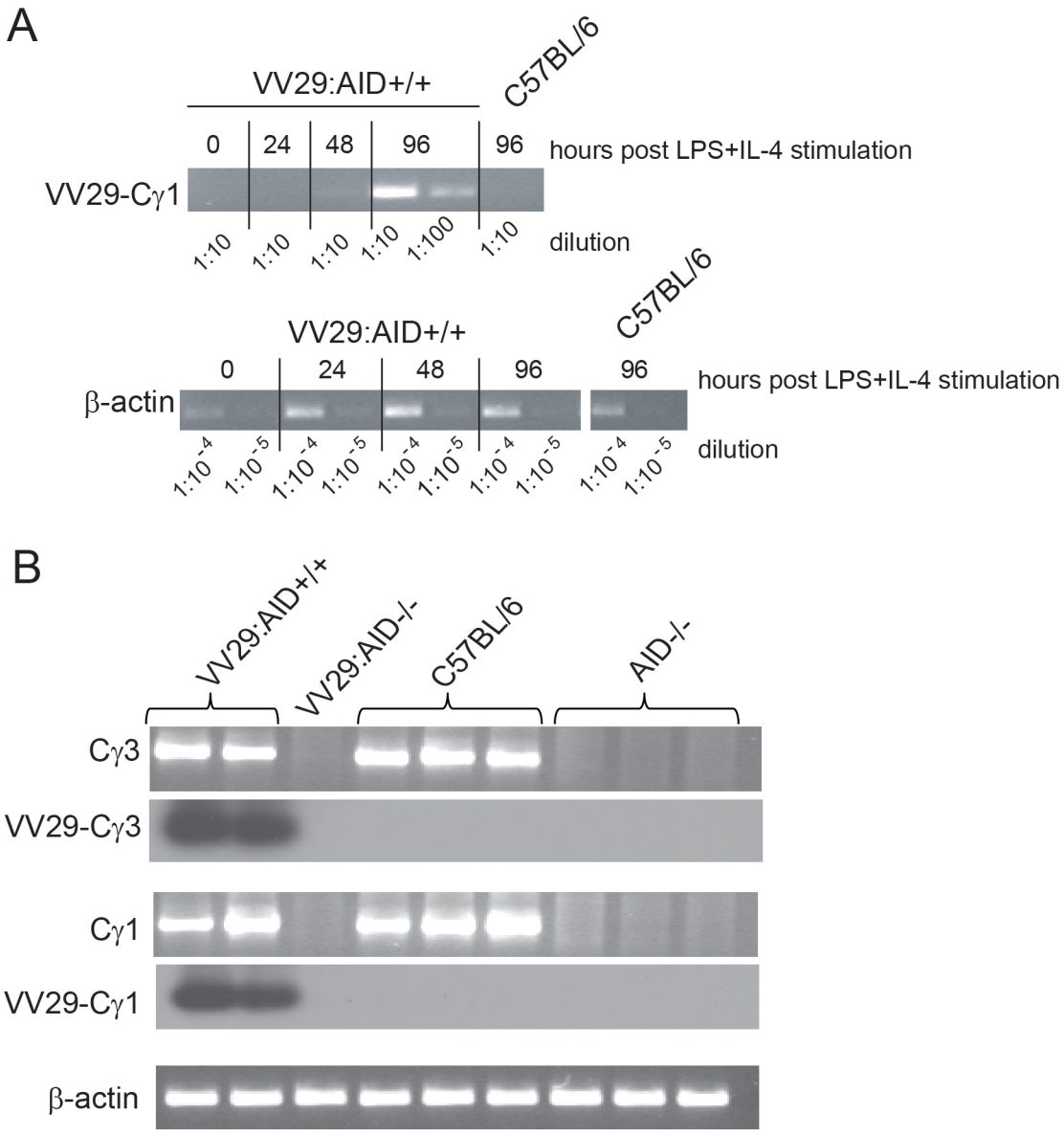
Because the VV29 transgene does not contain either the 3'RR or all the Igh locus sequences downstream the Cμ gene, translocation to the endogenous Igh locus is the only CSR mechanism to repair transgene Sμ AID-induced DNA damage. On the other hand, in the Δ3'RR transgene the presence of all of Igh locus S regions together with their surrounding sequences might lead to abortive downstream intrachromosomal CSR processes that compete with the interchromosomal translocation. Based on my findings, together with previous studies, and the fact that the frequencies of *in vitro* interchromosomal translocation in the VV29 B-cells are orders of magnitude higher than c-myc/Igh translocations frequencies (161) yet comparable to the frequencies of interallelic CSR among endogenous Igh loci (335), I conclude that interchromosomal translocations involving the Igh locus occur by an AID-mediated CSR mechanism and occur more often between chromosomes that share Igh associated regulatory elements. It would be interesting to determine whether the presence of a switch region or Igh enhancer elements near the c-myc gene would increase the frequency of translocations to the Igh locus.

In VV29 B-cells that are undergoing CSR, I can only find VV29 VDJ regions expressed with the VV29 transgenic Cμ gene and not the endogenous Cμ gene, even though I can easily detect expression of the VV29 region with endogenous Cγ regions. These results indicate that VV29 transgene translocations into the Igh locus do not involve trans-switching between the transgene Sμ and endogenous Sμ regions, implying that Sμ regions may be

differentially regulated from downstream S regions, perhaps to give directionality to the CSR machinery. One source of regulation may be chromosomal looping that associates the intronic Eμ enhancer with the downstream 3'RR enhancers during CSR (250). It is possible that DNA looping or protein complexes block Sμ regions from recombining with their chromosomal homologues. On the other hand, the DNA looping structure could leave downstream S regions more exposed to participate in interchromosomal recombination. To our knowledge, this is the first study that has indicated that two homologous Sμ regions do not recombine via trans-switching. These results indicate that there may have been evolutionary pressure to prevent recombination between the Sμ regions in order to minimize non-effective switch events and maximize the diversity of B-cell isotype responses to antigenic stimulation. Further studies are needed to determine the mechanism of regulation that inhibits Sμ to Sμ trans-recombination and whether translocations between other downstream S regions are also under similar regulation. Such regulation could also imply that it might be possible to manipulate the capacity of a DNA sequence to act as a site of chromosomal recombination and translocation.

Taken together, my results indicate that upon B-cell stimulation, multiple AID-induced pathways can be activated that can lead to DNA recombination events involving both cis- and trans-CSR and that these processes appear to be regulated to maximize the diversity of B-cell responses to antigens.

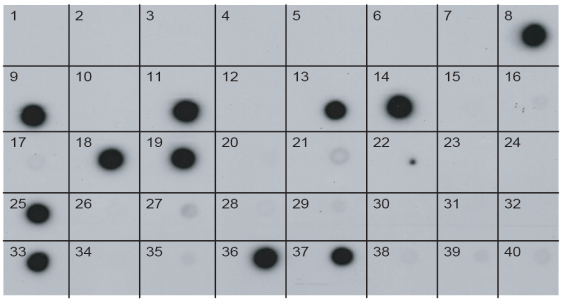
4. Appendix Figures



Appendix Figure 1

Appendix Figure 1. *In vitro* interchromosomal CSR. (A) Top: B-cells were either unstimulated (time 0h) or stimulated with LPS and IL-4 for the time periods indicated. Semi-quantitative PCR was performed with a transgene-specific forward primer (TND) and a Cγ1 reverse primer (8) to amplify VV29-Cγ1 transcripts. Bottom: Semi-quantitative PCR for β-actin was performed as loading control. **(B)** B-cells were isolated from the indicated mice and stimulated for 96 hours to undergo CSR. RT-PCR/Southern blot assay was performed to amplify Cγ3 or Cγ1 transcripts with primers described elsewhere (8). Transgenic transcripts (VV29-Cγ3 or VV29-Cγ1) were identified by hybridizing PCR products to TND probe. Each lane represents data from a single mouse. The β-actin PCR serves as loading control.

A



B

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L3RI primer ctcttcctcctgtcagtaac
V-gene #1   ctcttcctcctgtcagtaac
V-gene #2   ctcttcctcctgtcagtaac
V-gene #3   ctcttcctcctgtcagtaac
V-gene #4   ctcttcctcctgtcagtaac
V-gene #5   ctcttcctcctgtcagtaac
V-gene #6   ctcttcctcctgtcagtaac
V-gene #7   ctcttcctcctgtcagtaac
V-gene #8   ctcttcctcctgtcagtaac
V-gene #9   ctcttcctccggtcagtaac
V-gene #10  atcttcatccggttttgtaag
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Appendix Figure 2. Dot blot assay. (A) Dot blot assay of B-cells isolated from the spleen and stimulated with LPS and IL-4 for 4 days. RT-PCR was performed to amplify Cγ1 transcripts using a forward primer (L3RI), which hybridizes to Leader exon regions, and a reverse Cγ1 primer, which is located in exon 2 of the Cγ1 gene. The PCR products were cloned and 40 clones were transferred onto a nylon membrane and hybridized with a transgene-specific probe (TND) to identify VV29-Cγ1 clones. Eleven clones out of a total of 40 clones hybridized to the TND probe (27.5% of clones were VV29-Cγ1 transcripts). **(B)** In order to estimate the translocation frequency, the TND-negative clones were sequenced to determine the number of endogenous V-genes that are amplified using the forward L3RI primer. About 10 endogenous V-genes were identified (*Mus musculus Igh* locus on chromosome 12, Accession number: NG_005838). As shown, 8 of the V-genes have 100% homology to the L3RI primer, indicating that the primers are not biased to the transgene VDJ regions.

CHAPTER 4

AID-induced mutagenesis occurs in an immunoglobulin transgene that is missing Igh regulatory *cis* elements downstream of the Cmu gene

1. Introduction

During B-cell diversification, activation-induced cytidine deaminase (AID) initiates mutagenic processes such as somatic hypermutation (SHM) and immunoglobulin (Ig) gene conversion (Ig V-gene conversion) by deaminating cytosines to uracils, which are then repaired in an error-prone manner by Ung and Msh2 proteins (3, 7, 8, 22, 26). Ironically, the DNA repair pathways that are functional during SHM are also essential in protecting the genome from deleterious mutagenesis. For example, both Ung and Msh2 play an important role in repairing spontaneous DNA damage that can arise during DNA replication (95, 375). Furthermore, Ung and Msh2 are essential for the error-free repair of AID-induced DNA damage at non-Ig genes during B-cell activation (109). The regulatory pathways that promote the mutagenic function of Ung and Msh2 proteins at Ig loci and non-mutagenic function at non-Ig loci are not clear. It has been suggested that Ig *cis* elements may play an important role in AID targeting or in determining how AID-induced DNA damage is resolved. Among these *cis* elements are the 3' regulatory region (3'RR) enhancers, comprising a 30 kb region located downstream of the Cα gene, and the Igh intronic Eμ enhancer, located upstream

of the Sμ switch region, which may be essential in regulating AID activity (3, 4). Indeed, SHM is reduced in Ig transgenes missing 3'RR enhancers (178, 181), indicating that these *cis* elements may either be required to recruit AID, promote AID activity by allowing high rates of transcription, and/or act downstream of AID, recruiting error-prone factors to promote mutagenesis.

While, SHM appears to be important in all jawed vertebrates (376), however, there is substantial variation in the ability to undergo Ig V-gene conversion between species. In birds, horses, pigs, and rabbits, Ig V-gene conversion dominates V-gene diversification, whereas in sheep, mice and humans, Ig V-gene conversion activity is difficult to detect or may be absent (2). The basis for the variation in Ig V-gene conversion activity between species is not understood.

In mice that carry an Igh transgene that is optimized for detection of gene conversion, events that resemble Ig V-gene conversion are found (65, 66) suggesting that the mechanisms needed for Ig V-gene conversion are present in mouse B cells, but may be at low levels or repressed. However, Rad54 deficiency has a strong effect on chicken Ig V-gene conversion yet does not affect the Ig V-gene conversion observed in transgenic mice, raising a question of whether the two processes rely on the same mechanism (68). In particular, it is not known whether Ig V-gene conversion in transgenic mice requires AID.

In this study I have analyzed Ig V-gene conversion and SHM in the VV29 mice, which is not inserted into the Igh locus, and therefore, does not contain Ig *cis* elements downstream of the Cμ gene. I have used this system to investigate AID activity and Ung/Msh2 function at the transgene locus.

2. Results

2.1 Transgene V-gene conversion depends on interchromosomal isotype switching.

I wanted to determine whether the Ig V-gene conversion that was previously characterized in VV29 transgenic mice (66) is initiated by AID. Immunoglobulin V-gene conversion can be easily detected in VV29 mice because they carry a transgene construct containing two closely spaced tandem VDJ segments, where the upstream VDJ (2B4) is promoterless and the downstream VDJ (R16.7) has a functional promoter (Figure 1A). Previous studies have shown that upon immunization with Ars-KLH, expression of the 2B4 VDJ sequences are found in IgG mRNAs and result from sequence transfers from the 2B4 VDJ segment to the R16.7 VDJ segment. These DNA sequence transfers occur by a V-gene conversion mechanism and can easily be detected by RT-PCR/Southern blot assays using probes that are specific to the 2B4 VDJ regions (65, 66, 68).

To determine whether AID was required for transgene Ig V-gene conversion, I assayed VV29-Cμ transcripts because both AID-deficient and AID-sufficient VV29 mice are able to produce Cμ transcripts. Cμ transcripts were amplified from splenocytes of immunized VV29:AID$^{+/+}$, VV29:AID$^{+/-}$, VV29:AID$^{-/-}$, nontransgenic AID$^{-/-}$, and C57BL/6 mice. The amplified transcripts were analyzed by Southern blots with the 2B4 probe to identify Cμ transcripts that are

associated with transgene VDJ segments that have undergone Ig V-gene conversion, or with either the TND or R16.7 probes to identify all VV29-Cμ transcripts. As shown in Figure 1B, transgene Ig V-gene conversion events are not detected among VV29-Cμ transcripts regardless of whether or not AID was expressed in the splenocyte population. Similarly, Ig V-gene conversion events are not detected in blood lymphocytes (Figure 1C), or in primary immunized mice, where the levels of splenocyte transgene-derived Cμ transcripts are the highest (Figure 1D).

To further increase the sensitivity of detecting Ig V-gene conversion events, I reasoned that these events would be enriched in splenocytes responding to antigenic stimulus. Therefore, I sorted splenocytes from immunized VV29:AID$^{+/+}$, VV29:AID$^{+/-}$, and VV29:AID$^{-/-}$ transgenic mice into activated IgM+ cells by PNA (peanut agglutinin) staining to isolate activated germinal center IgM cell populations. Two sorted populations, IgM^{+} PNA^{high} and IgM^{high} PNA^{high}, were collected and RNA was extracted to assay for Ig V-gene conversion using the RT-PCR/Southern blot assay. Results in Figure 1E show that, although sorted IgM+ germinal center B-cells are expressing the VV29 transgene, as detected by the hybridization of the PCR products to the transgene-specific TND probe, no detectable Ig V-gene conversion is found in these germinal center IgM+ B-cells regardless of the presence or absence of AID.

The lack of Ig V-gene conversion events among transgenic VDJ-Cμ transcripts in immunized VV29 B-cells contrasts with the easily demonstrated Ig V-gene conversion events among transgenic VDJ-Cγ transcripts (Figure 1F, *Supplementary Figure 1A-B*, (66, 68)). Furthermore, I was able to detect Ig V-gene conversion in Cγ transcripts from cDNA that was diluted over a 10,000-fold range, indicating that the RT-PCR/Southern blot assay is quit sensitive (*Supplementary Figure 1C*). One main difference between transgenic VDJ-Cμ and transgenic VDJ-Cγ transcripts is that all transgenic VDJ-Cγ mRNAs arise from genes that have undergone interchromosomal translocation with the endogenous Igh locus, whereas transgenic VDJ-Cμ transcripts are derived from the VV29 transgenes that may have not undergone translocations (Chapter 3, (377)). These results suggest that Ig V-gene conversions in VV29 mice are linked to AID-dependent interchromosomal isotype switch events.

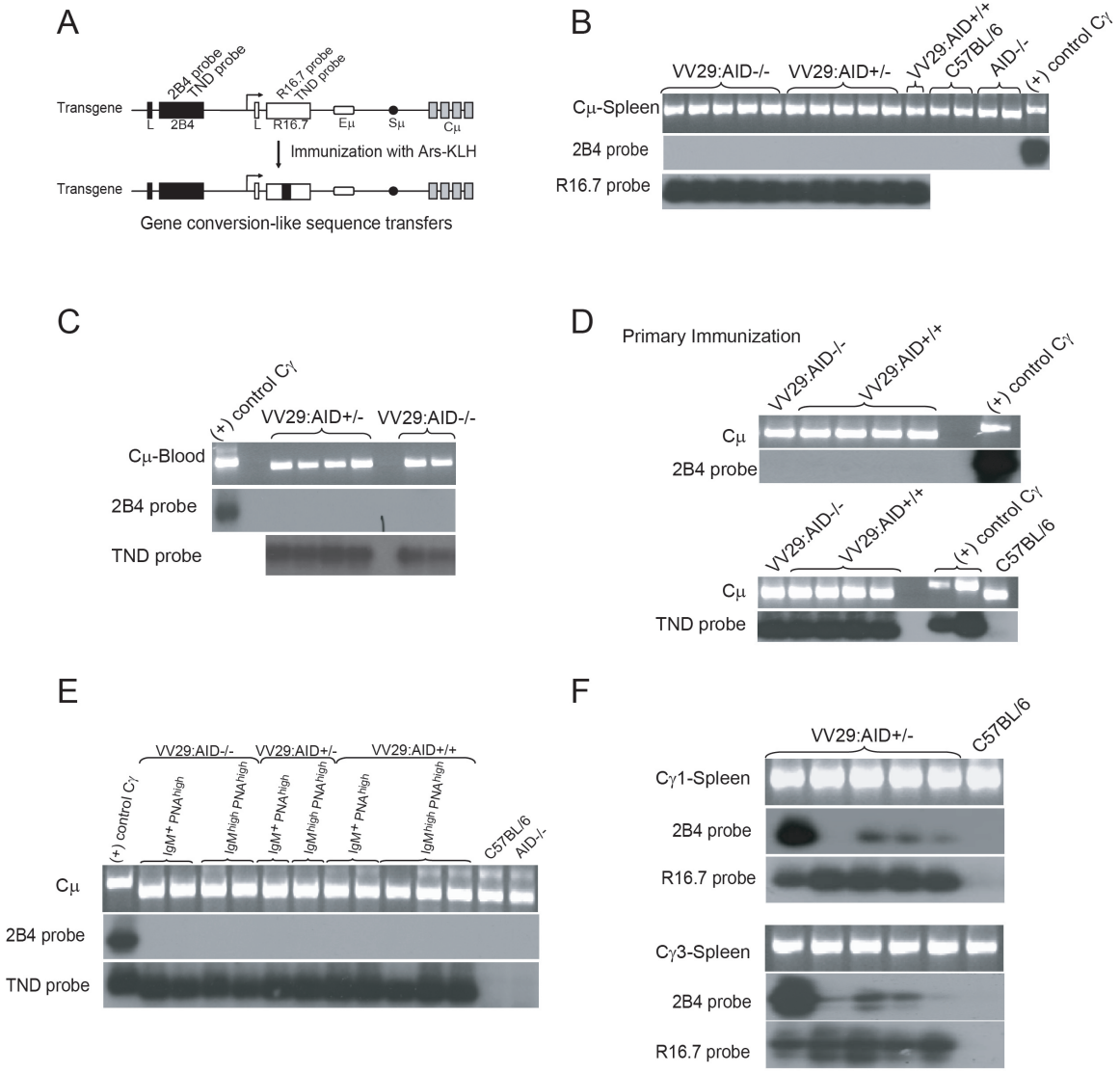

A
2B4 probe
TND probe
R16.7 probe
TND probe
Transgene
L
2B4
L
R16.7
Eμ
Sμ
Cμ
Immunization with Ars-KLH
Transgene
Gene conversion-like sequence transfers
B
VV29:AID-/-
VV29:AID+/-
VV29:AID+/+
C57BL/6
AID-/-
(+) control Cγ
Cμ-Spleen
2B4 probe
R16.7 probe
C
(+) control Cγ
VV29:AID+/-
VV29:AID-/-
Cμ-Blood
2B4 probe
TND probe
D
Primary Immunization
VV29:AID-/-
VV29:AID+/+
(+) control Cγ
Cμ
2B4 probe
VV29:AID-/-
VV29:AID+/+
(+) control Cγ
C57BL/6
Cμ
TND probe
E
VV29:AID-/-
VV29:AID+/-
VV29:AID+/+
(+) control Cγ
IgM+ PNAhigh
IgMhigh PNAhigh
IgM+ PNAhigh
IgMhigh PNAhigh
IgM+ PNAhigh
IgMhigh PNAhigh
C57BL/6
AID-/-
Cμ
2B4 probe
TND probe
F
VV29:AID+/-
C57BL/6
Cγ1-Spleen
2B4 probe
R16.7 probe
Cγ3-Spleen
2B4 probe
R16.7 probe

Figure 1

Figure 1. Transgene V-gene conversion depends on interchromosomal isotype switching. (A) Schematic diagram of Ig gene conversion in VV29 mice. The VV29 consists of two closely spaced tandem VDJ segments (2B4 and R16.7), the Eμ intronic enhancer, a 600-bp Sμ switch region, and a Cμ heavy chain. The two VDJ segments respond to the Ars hapten. The first VDJ (2B4) is promoterless, whereas the second VDJ (R16.7), situated 1.5 kb downstream, has a promoter (black arrow). Upon immunization with Ars-KLH, DNA sequences from the promoterless 2B4 VDJ segment are transferred into the R16.7 VDJ region which is detected by RT-PCR/Southern blot assays with the 2B4 probe. The location of the TND, 2B4, and R16.7 probes are shown. Since the 2B4 VDJ segment lacks a promoter, expression of VDJ segments that hybridize to the 2B4 probe are a result of Ig V-gene conversion events. L; leader exon. Diagram not drawn to scale. **(B)** The indicated mice were immunized with Ars-KLH and RNA was isolated from splenocytes for amplification for Cμ transcripts by RT-PCR. The PCR products were transferred onto two different nylon membranes and hybridized to the 2B4 probe to identify Ig V-gene conversion events, or to the R16.7 probe to identify all VV29-Cμ transcripts. Total splenocytes from C57BL/6 and $AID^{-/-}$ mice serve as negative controls. Each lane represents data from a single mouse. The positive control is a plasmid that contains a Cγ transcript that is associated with the gene converted VV29 VDJ segment. **(C)** The same RT-PCR assay as described in (B) from blood lymphocytes. The PCR products were hybridized to the 2B4 probe to identify Ig V-gene conversion events or to the TND probe to identify all VV29-Cμ

transcripts. Each lane represents data from a single mouse. **(D)** Mice received one dose of immunization before splenocytes were harvested to isolate RNA and perform RT-PCR/Southern blot assays as described in (B). **(E)** The same RT-PCR assay as described in (B) with activated IgM+ B-cells isolated from splenocytes. Total splenocytes from C57BL/6 and $AID^{-/-}$ mice serve as negative controls. Each lane represents data from a single mouse. **(F)** The indicated mice were immunized as described in (B) and $C\gamma1$ or $C\gamma3$ transcripts were amplified. Each lane represents data from a single mouse.

2.2 AID activity is lower at the transgene locus.

The inability to undergo Ig V-gene conversion at the transgene locus could be due to inability of the transgene VDJ region to undergo sufficient AID-induced DNA damage. The VV29 transgene is missing Igh *cis* elements downstream of the Cμ gene, therefore, it is possible that the transgene VDJ region is unable to recruit AID to a threshold level required to induce gene conversion. In order to determine AID activity at the transgene locus, I assayed for SHM at the transgene V-region in transgenic VV29:AID$^{+/+}$ mice (VV29:wildtype) and in AID-deficient transgenic VV29 mice (VV29:AID$^{-/-}$). Splenocytes were harvested from immunized mice one week post the second immunization with Ars-KLH and activated B-cells were isolated by sorting for the B220^{+}PNA^{high} population. To make sure that the VV29 transgene had not undergone a translocation into the Igh locus, I isolated RNA from the sorted B-cells and after RT-PCR, used the cDNA to amplify both endogenous and transgene VDJ-Cμ transcripts to look for SHM at the V-region and verify that the transgene VDJ region was associated with the transgene Cμ gene and the endogenous VDJ region was associated with the endogenous Cμ gene. Due to allotypic differences between the two Cμ genes, by sequencing analysis, I was able to distinguish the endogenous Cμ gene from transgene Cμ gene (Chapter 3, (377)). A total of 251 clones from VV29:wildtype mice and a total of 119 clones from VV29:AID$^{-/-}$ mice were analyzed for SHM by sequencing analyses. Transgene V-regions from VV29:wildtype mice had a significantly higher mutation frequency (9 x 10^{-4}),

about 7 fold higher, than transgene V-regions from VV29:AID$^{-/-}$ mice (1.3 x 10^{-4}) (Figure 2A). This indicates that the transgene VDJ locus is capable of accruing AID-dependent mutations.

Comparison of transgene V-region with endogenous V-region in VV29:wildtype mice, however, showed that AID is not as efficiently targeted or active at the transgene locus as the mutation frequency at the endogenous locus (42 x 10^{-4}) was significantly higher, about 5 fold, than the transgene locus (Figure 2B). The lower mutation frequency at the transgene locus is not likely due to inefficient transcription of the transgene, as the levels of transcripts between the VV29 VDJ-Cμ at the transgene locus and the transgene VDJ that have undergone a translocation to the endogenous Igh locus, VV29 VDJ-Cγ transcripts, are comparable (*Supplementary Figure 2A*). However, the mutation frequency is about 8-fold higher (VV29-Cγ: 70 x 10^{-4}) at the translocated transgene VDJ region (*Supplementary Figure 2B*). It should be noted, however, that the VV29-Cγ mutation frequency was determined from only 4 clones and that this frequency may change if more clones are analyzed. Nevertheless, these results indicate that the transgene is probably missing regulatory *cis* elements that are necessary for efficient mutagenesis.

In order to investigate whether the lower SHM frequency at the transgene V-region is due to inefficient AID activity, I performed the same RT-PCR/sequencing analysis with VV29:Ung$^{-/-}$:Msh2$^{-/-}$ double knockout B-cells to

test for AID activity (footprints). I compared the mutation frequencies at the transgene locus and the endogenous locus in VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice. A total of 180 clones from the transgene locus, and 156 clones from the endogenous locus of VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice were sequenced. The mutation frequency at the transgene locus was 3 x 10^{-4}, which is about 5 fold lower when compared to the mutation frequency at endogenous locus (17 x 10^{-4}) (Figure 2C). This indicates that AID is either not properly recruited to the transgene locus or is not as active at the transgene locus.

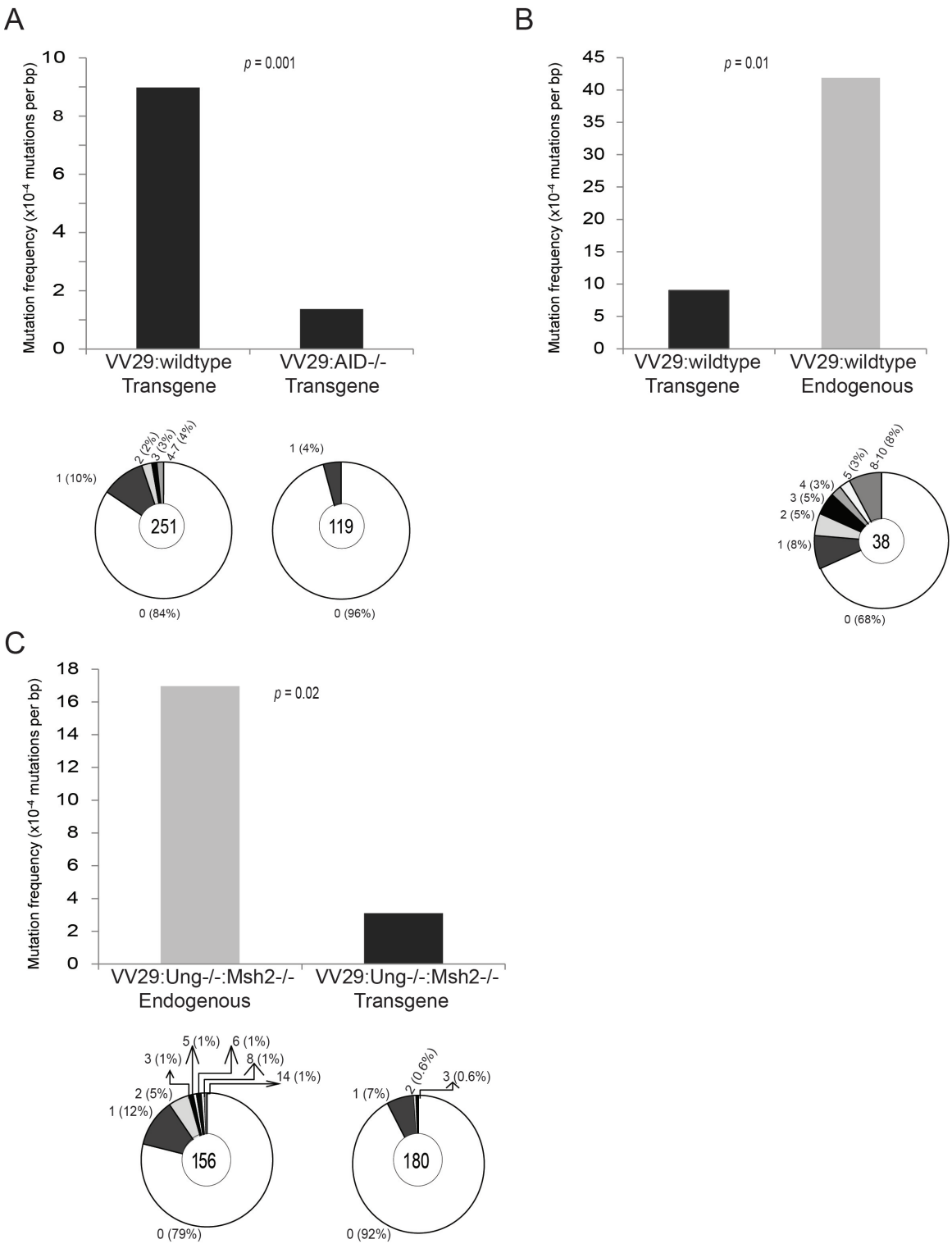

A
Mutation frequency (x10-4 mutations per bp)
p = 0.001
VV29:wildtype Transgene
VV29:AID-/- Transgene
1 (10%)
2 (2%)
3 (3%)
4-7 (4%)
251
0 (84%)
1 (4%)
119
0 (96%)
B
Mutation frequency (x10-4 mutations per bp)
p = 0.01
VV29:wildtype Transgene
VV29:wildtype Endogenous
8-10 (8%)
5 (3%)
4 (3%)
3 (5%)
2 (5%)
1 (8%)
38
0 (68%)
C
Mutation frequency (x10-4 mutations per bp)
p = 0.02
VV29:Ung-/-:Msh2-/- Endogenous
VV29:Ung-/-:Msh2-/- Transgene
5 (1%)
6 (1%)
3 (1%)
8 (1%)
14 (1%)
2 (5%)
1 (12%)
156
0 (79%)
2 (0.6%)
3 (0.6%)
1 (7%)
180
0 (92%)

Figure 2

Figure 2. AID activity is lower at the transgene locus. (A) Top: SHM frequency was determined from activated B-cells that were isolated from splenocytes of immunized wildtype VV29 transgenic mice (VV29:wildtype) and VV29:AID$^{-/-}$ mice. RNA was isolated and RT-PCR was performed to amplify Cμ transcripts. The PCR products were cloned and sequenced to identify transgenic Cμ transcripts that were associated with transgenic VDJ regions. The 301-bp V-region was analyzed for SHM. Mutation frequency was determined by dividing the total number of mutations by the total number of bp analyzed. Clones with identical mutations, and clones that shared mutations were considered identical and counted once. A total of 2 independent experiments were performed with 4 mice per experiment for VV29:wildtype mice and 1 experiment was performed with 4 mice per experiment for VV29:AID$^{-/-}$ mice. Bar graphs for VV29:AID$^{+/+}$ represent the combined data from the two independent experiments. *p*-value was calculated by Mann-Whitney test. Bottom: Pie charts representing SHM data. The center of the pie chart indicates the total number of clones sequenced. The number around the pie chart represents the number of mutations. The numbers inside the parenthesis are the percentages of clones containing the indicated number of mutations. **(B)** Top: The same data for VV29:wildtype transgene as in (A) is presented. Comparison is made to the endogenous VDJ-Cμ transcripts. NCBI Blast engine was used to compare the cloned endogenous V-region to germline V-regions. SHM was determined as described in (A). A total of 2 independent experiments were performed with 4 mice per genotype per experiment. Bar graphs represent the combined data from two independent

experiments. *p*-value was calculated by one sample Student's *t*-tests. Bottom: Same as described in (A). **(C)** Top: SHM frequencies are presented as described above. A total of 2 independent experiments were performed with 4 mice per genotype per experiment. Bar graphs represent the combined data from two independent experiments. *p*-value was calculated by one sample Student's *t*-tests. Bottom: Same as described in (A).

2.3 Ung and Msh2 have a mutagenic function at the transgene locus.

Comparison of the mutation frequencies in VDJ-Cμ transcripts between the endogenous Igh locus from VV29:wildtype mice (42 x 10^{-4}) and the endogenous Igh locus from VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice (17 x 10^{-4}) shows a 2.5 fold decrease in the mutation frequency in VV29Ung$^{-/-}$:Msh2$^{-/-}$ mice (Figure 3A). Similarly, comparing the mutation frequency at the transgene locus of VV29:wildtype mice (9 x 10^{-4}) to the mutation frequency at the transgene locus in VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice (3 x 10^{-4}), shows a 3 fold lower mutation frequency in the VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice. Transition and transversion mutations between transgene and endogenous loci in VV29:wildtype mice were comparable (Figure 3B). These results indicate that Igh regulatory *cis* elements downstream of the Cμ gene are not necessary for inducing the mutagenic function of Ung and Msh2 proteins.

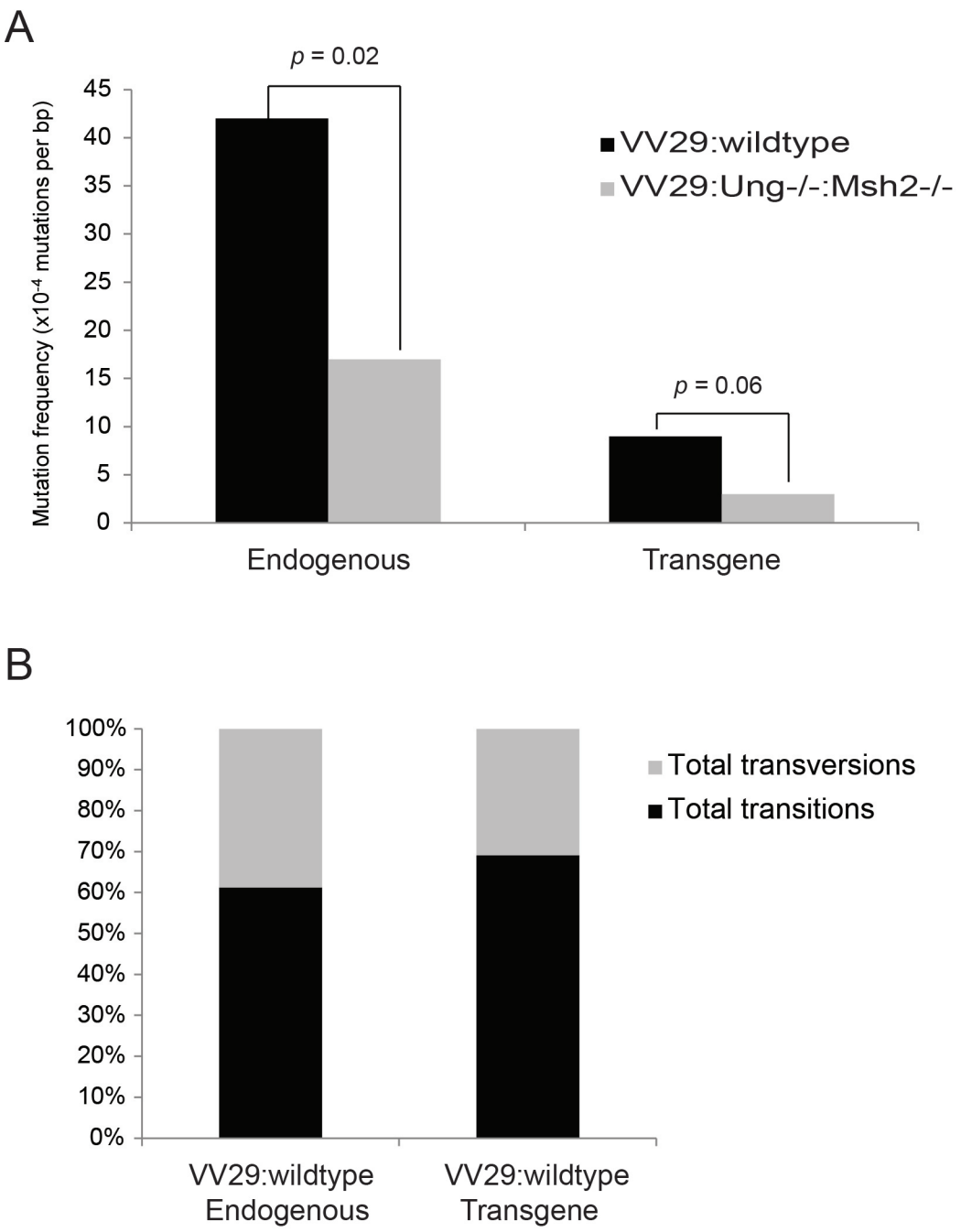
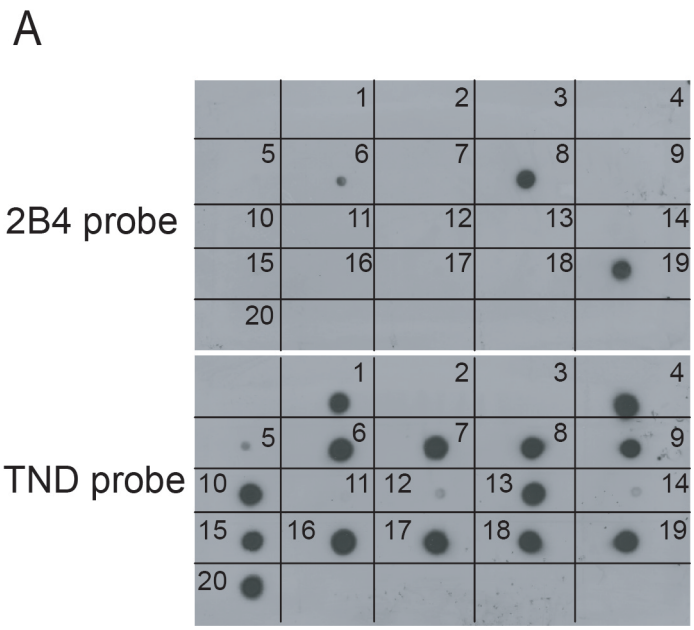

A
p = 0.02
p = 0.06
Mutation frequency (x10-4 mutations per bp)
45
40
35
30
25
20
15
10
5
0
VV29:wildtype
VV29:Ung-/-:Msh2-/-
Endogenous
Transgene
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Total transversions
Total transitions
VV29:wildtype Endogenous
VV29:wildtype Transgene

Figure 3

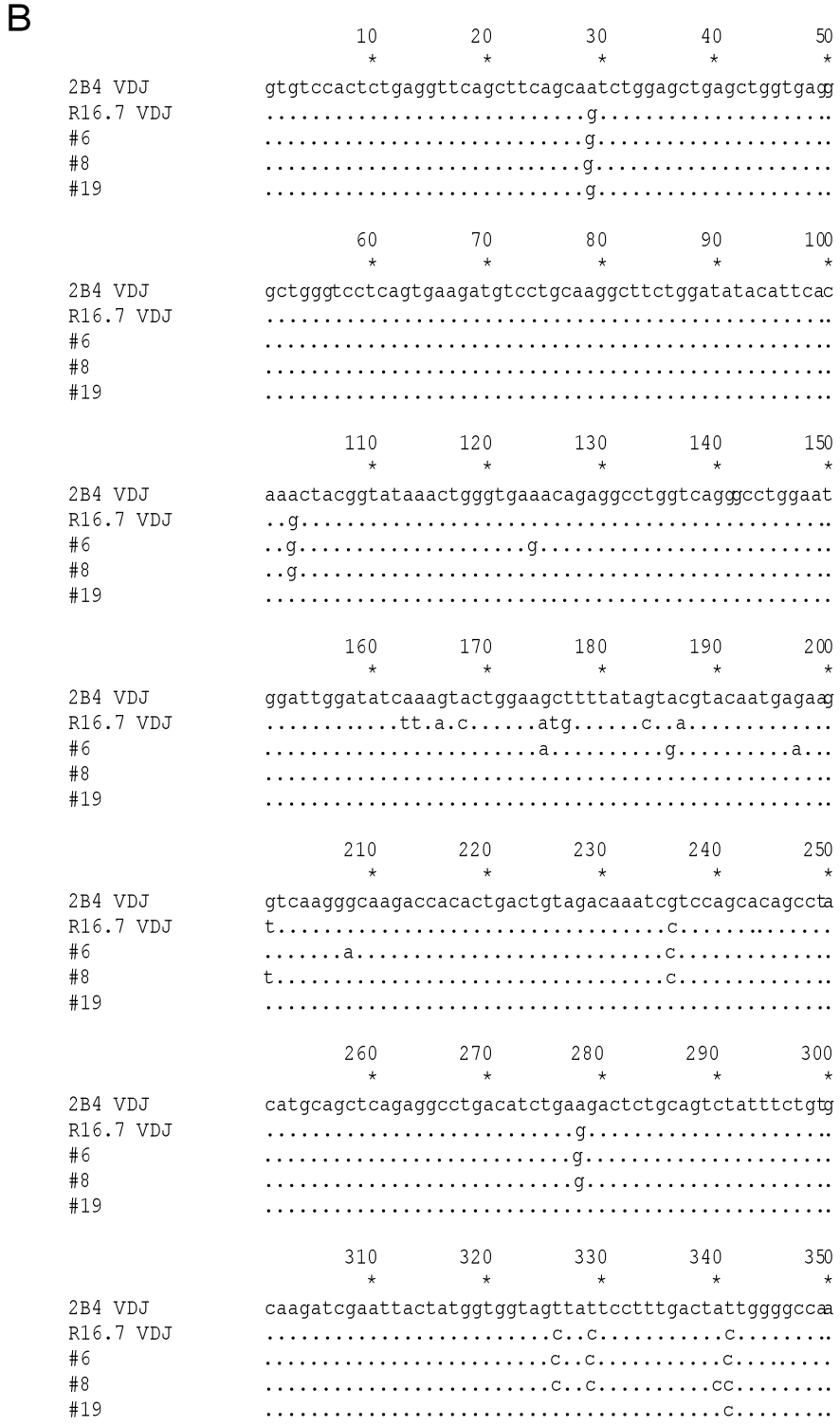
Figure 3. Ung and Msh2 have a mutagenic function at the transgene locus. **(A)** The same data for VV29:wildtype transgene locus (see Figure 2A), VV29:wildtpe endogenous locus (see Figure 2B), VV29:$Ung^{-/-}$:$Msh2^{-/-}$ transgene locus and endogenous locus (see Figure 2C) are presented here. The 301-bp V-region was analyzed for SHM. Mutation frequency was determined by dividing the total number of mutations by the total number of bp analyzed. Clones with identical mutations, and clones that shared mutations were considered identical and counted once. NCBI Blast engine was used to compare the cloned endogenous V-region to germline V-regions. A total of 2 independent experiments were performed with 4 mice per genotype per experiment. Bar graphs represent the combined data from two independent experiments. *p*-values were calculated by one sample Student's *t*-tests. **(B)** Percent of total transversion and transition mutations.

3. Supplementary Data



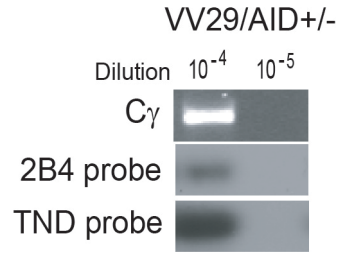
Supplementary Figure 1A

B



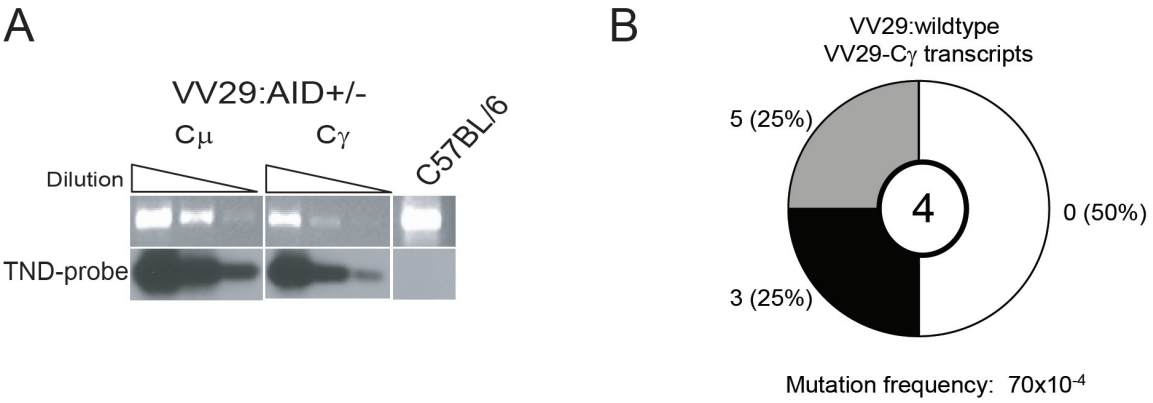
Supplementary Figure 1B

C



Supplementary Figure 1C

Supplementary Figure 1. Sensitivity of RT-PCR/Southern blot analysis. (A) PCR products from Cγ transcripts that are associated with transgenic VDJ regions were cloned and transferred onto nylon membranes to hybridize to top: 2B4 probe to identify gene conversion events, and bottom: TND probe to identify all VV29 transgene transcripts. A total of 20 clones were hybridized and gene conversion was detected in 3 clones (clones 6, 8, and 19). **(B)** Clones 6, 8, and 19 were sequenced to confirm the gene conversion events. Dots represent no change in sequence. **(C)** The cDNA from an immunized VV29:AID$^{+/-}$ mouse that was previously shown to have undergone Ig V-gene conversion was diluted as indicated and Cγ transcripts were PCR amplified. Southern blot assays were performed with the 2B4 probe to identify VV29-Cγ transcripts that have undergone Ig V-gene conversion events, and the TND probe to identify all VV29-Cγ transcripts.



Supplementary Figure 2. Transcript levels of VV29-Cμ and VV29-Cγ. (A) Semi-quantitative RT-PCR/Southern blot hybridization of splenocytes from fully immunized mice as indicated in the figure. The cDNA for VV29:AID$^{+/-}$ mice was serially diluted 1:10, 1:100, and 1:1000 and PCR was performed with the L3RI forward primer and either a Cμ primer or a Cγ reverse primer (hybridizes to Cγ1, Cγ2a, Cγ2b). The PCR products were hybridized to the transgene-specific probe, TND. All of the samples are from the same gel. **(B)** The mutation frequency was determined as described in figure 2A with the same (VV29:wildtype) mice that were used in figure 2A.

4. Discussion

Lack of Ig V-gene conversion at the transgene locus may be due to low levels of AID activity.

The VV29 transgenic mouse, composed of two VDJ segments, the Eμ intronic enhancer, a 600 bp Sμ region, and the Cμ constant gene, was designed to optimize the detection of Ig V-gene conversion in mice (66, 68). Here, I wanted to determine whether mouse Ig V-gene conversion was dependent on AID activity. My results show that Ig V-gene conversion is only detected in VV29 VDJ segments that have translocated into the endogenous Igh locus. Because I have shown that translocation depends on AID (Chapter 3, (377)) I am concluding that Ig V-gene conversion in mice, within the VV29 transgene context, is at least indirectly AID-dependent, although it is possible that AID would have a direct involvement in gene conversion at the endogenous locus post the translocation event.

I could not detect Ig V-gene conversion in VV29-Cμ transcripts from fully immunized mice by RT-PCR/Southern blot assays, although the assay is relatively sensitive. For example, I showed that 15% (3 clones out of 20 total clones analyzed, *Supplementary Figure 1A*) of VV29-Cγ transcripts have undergone gene conversion and we were still able to detect a positive gene conversion signal in VV29-Cγ transcripts by the RT-PCR/Southern blot assay at

10,000 fold dilution (*Supplementary Figure 1B*). Furthermore, I did not detect Ig V-gene conversion in any of the 251 transgene VDJ-Cμ clones from VV29:AID$^{+/+}$ mice that were analyzed by sequencing (data not shown). Moreover, the gene-converted transgenic VDJ segment (2B4) has a two-fold higher affinity for the immunized antigen than the non-converted VDJ segment (R16.7) (65, 67). Therefore, antigenic selection should have increased the presence of the gene converted transcripts and I should have been able to detect them in the RT-PCR/Southern blot assays if they were present. Based on these results, I conclude that Ig V-gene conversion, at the transgene locus, occurs extremely infrequently, or is linked to CSR.

The inability to detect Ig V-gene conversion in VDJ-Cμ transcripts in mouse B-cells contrasts with the ability to detect such transcripts in chickens (378). This raises the question of whether mice generate Ig diversity by gene conversion. Strong evidence for Ig V-gene conversion occurring in mice is lacking and reports that AID-induced DNA damage is repaired in G1 of the cell cycle (273) make it further difficult to hypothesize that Ig V-gene conversion plays a role in mouse or human B-cell diversification because the conversion pathway is reportedly not active during G1 (278, 279). However, recently it was shown that deletion of the *Xrcc2* gene, which is part of the homologous recombination pathway, resulted in more DNA breaks (genomic instability), decreased CSR to IgG1, and a proliferation defect (64), indicating that by promoting genomic stability, gene conversion may, at least, play an indirect role during B-cell

diversification of mice and humans. Therefore, the contribution of homologous recombination and gene conversion among endogenous Ig genes needs to be further investigated.

The inability to detect Ig V-gene conversion at the transgene locus could also indicate that the transgene is inefficient in some function required for gene conversion. For example, AID recruitment/activity may not be sufficient to initiate the gene conversion pathway. Indeed, when I tested for AID activity at the transgene locus, I showed that, although the transgene VDJ region is capable of accumulating AID-induced mutations, the mutation frequency is lower than the endogenous Igh locus. The lower mutation frequency is likely due to lower AID activity at the transgene locus because VV29:$Ung^{-/-}$:$Msh2^{-/-}$ mice have lower mutations, i.e. AID footprints, at the transgene locus when compared to the endogenous locus. The background mutation frequency, as indicated by VV29:$AID^{-/-}$ mice, is $1.3x10^{-4}$ which is comparable to the PCR error-rate I have seen before (Chapter 5, (379)). These results imply that AID activity is less than optimal at the transgene locus and may reflect that the VV29 transgene is missing 3'RR elements that have previously been implicated to be important for AID activity at transgenic VDJ regions (165, 178, 181).

Ung and Msh2 have a mutagenic function at the transgene locus.

It has been suggested that the Igh 3'RR enhancers are either important in AID recruitment and/or promote error-prone repair post AID-induced deamination (3). My results indicate that an Ig transgene that is missing all of the Igh regulatory *cis* elements downstream of the Cμ gene can accumulate AID-induced mutations. These mutations, similar to the endogenous V-regions, are dependent on the function of Ung and Msh2 proteins. The mutagenic function of Ung and Msh2 at the transgene locus contrasts with the role of these proteins in promoting error-free repair of AID-induced DNA damage of genes located on non-Ig loci (109). These results indicate that the VV29 transgene, although it is missing Igh 3'RR enhancers, contains the necessary Igh *cis* elements needed to promote the mutagenic function of Ung and Msh2.

Although, deletion of both Ung and Msh2 result in a reduction of mutations at the Igh V-regions (109), at the Ig switch regions, the mutation frequency is enhanced in Ung/Msh2 double knockout mice (380). These results may indicate that Ung and Msh2 have different functions at different Ig loci. However, an alternative explanation may be that since CSR is dramatically reduced in Ung/Msh2 double knockout mice (381), switch regions that are targeted by AID, which are usually excised out during recombination remain, and are replicated over by error-prone polymerases, thus enhancing mutagenesis at the switch regions. It is, however, also possible that Ung and Msh2 are both mutagenic and

protective. In a recent study, it was shown that Msh2 and Msh6 have both a protective and a mutagenic functions at the Igh V-regions (382). In this study, it was suggested that the balance between error-prone factors and error-free factors may determine the mutagenicity of Msh2 and Msh6 (382). However, it still unclear how this is regulated and which factors are essential.

It should be noted that it is likely that the mutagenic function of Ung and Msh2 are influenced by the chromosomal location of the transgene. I have previously shown that the VV29 transgene is not inserted on chromosome 12 which contains the Igh locus (Chapter 3, (377)). And because the VV29 transgene is inserted on a relatively large chromosome, it does not appear that VV29 is influenced by the Igλ locus, which is located on the much smaller chromosome 16 (Chapter 3, (377)). However, I cannot confidently rule out the possibility that the VV29 transgene is on chromosome 6 which contains the Igκ locus (Chapter 3, (377)). Transgenes that are similar to the VV29 transgene and that are located on chromosome 5 have also been shown to accumulate mutations (383) indicating that chromosomal location may not influence the VV29 transgene. However, there is a possibility that the chromosome location may have some influence in affecting mutagenesis of the VV29 transgene. Therefore, it is possible that my conclusion may be biased, if by chance, the VV29 transgene has integrated into a chromosome that is localized at a particular space in the nucleus, or has some DNA element that preferentially recruit AID. In order to test whether chromosomal integration site influences mutagenesis at

the VV29 transgene, several transgene, integrated at different chromosomal locations, need to be investigated.

Because the VV29 transgene resembles the Igh locus in terms of containing Ig promoters, the Eμ enhancer, and a highly repetitive 600 bp Sμ region (AID hotspots), I suggest that the mutagenic function of Ung and Msh2 are likely due to the higher frequency of AID deamination at the transgene locus as compared to other non-Ig genes that have been shown to be targeted by AID (109). Therefore, it is quite possible that higher AID deamination at Ig loci leads to repeated excisions of the uracils by Ung and Msh2 pathways, leaving extended DNA breaks which can signal the cell to either repair the DNA damage before replication (S phase) or undergo cell death due to the inability to cope with such extensive DNA damage. At non-Ig loci, the lower level of AID-mediated deamination would generate fewer DNA damage sites, which could be tolerated by the cells and fixed at a later cell cycle stage, i.e. early S phase or G2 phase, or alternatively, the lower levels of DNA damage could be repaired quickly by the error-free machinery in G1.

I suggest that the mechanism of resolving AID-induced DNA damages at the swtich regions may be different, due to the high number of AID hotspots, which unlike V-regions, result in DSBs. These DSBs are more likely to be resolved by the recombination process because other DNA factors, such as the NHEJ proteins are more likely to bind to DSBs and initiate CSR.

I propose that there may be a limit to the extent of DNA damage that promotes error-free repair, and any additional DNA damage beyond this limit would activate error-prone DNA repair. Therefore, the level of DNA damage may be at least one of the mechanisms which promotes SHM at the V-regions, although, I do not exclude the possibility that error-prone factors could also be activated at Ig loci independently of the amount of DNA damage by a complementary mechanism. The hypothesis that level of DNA damage controls error-prone repair is not novel as studies in bacteria have shown that the extent of DNA damage can dictate whether the damage is repaired in an error-free or error-prone manner (362, 384). Here, I suggest that perhaps the amount of DNA damage may also determine when and how repair happens during B-cell diversification.

CHAPTER 5

p21 is dispensable for AID-mediated class switch recombination and mutagenesis of immunoglobulin genes during somatic hypermutation

Maryam Shansab, Erik Selsing

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1. Introduction

In B cells, activation-induced cytidine deaminase (AID) induces somatic hypermutation (SHM) at rearranged immunoglobulin (Ig) variable (V) regions (3). Previous studies have shown that both monoubiquitination of proliferating cell nuclear antigen (PCNA) and translesional DNA polymerase activity are important for inducing mutagenesis during SHM (120, 121). Regulation of PCNA ubiquitination by p21, also known as Cdkn1a and $p21^{Cip1/Waf1}$, is an important mechanism that controls mutation loads in mammalian cells (130, 131).

The role of p21 in B cells is not clear. Studies have suggested that inactivating p21 during B cell activation may be essential to allow proper cell cycle progression (155, 156). However, because p21 can also function as a negative regulator of mutagenesis by modulating PCNA ubiquitination, it is unclear whether p21 inactivation in B cells is essential for promoting SHM or preventing cell cycle arrest. I have now investigated the role of p21 during SHM. I have tested whether p21 deficient (*Cdkn1a*$^{-/-}$) B cells displayed alterations in SHM, specifically increases in mutations at A:T bp which would be predicted if p21 functioned as a negative regulator of error-prone translesional polymerases. Furthermore, I have tested whether p21 regulation during B cell activation was dependent on AID-induced DNA damage.

2. Results

2.1 p21 expression is not regulated by AID.

First, I examined the expression pattern of p21 during B cell activation, and also assessed whether AID expression affected the p21 expression pattern, by performing a time-course experiment to monitor p21 mRNA levels at different time points. B cells from wildtype (*Aicda*$^{+/+}$) or AID-deficient (*Aicda*$^{-/-}$) mice were either unstimulated or stimulated with LPS and IL-4 for 8, 24, 48, 72, or 96 hours (hr), and then p21 mRNA levels were determined by quantitative Real-time PCR. As shown in Figure 1, p21 mRNA levels sharply increase 50-65 fold at 8 hours after B cell stimulation and then drop to near unstimulated levels at 24 hours post stimulation. Subsequently, p21 expression increases to 13-18 fold at 48 and 72 hours post stimulation, reaching a maximum of 25 fold at 96 hours post stimulation. I did not detect any significant difference in p21 expression levels between *Aicda*$^{+/+}$ and *Aicda*$^{-/-}$ B cells. These data indicate that the pattern of p21 expression levels in stimulated B cells are regulated independent of AID.

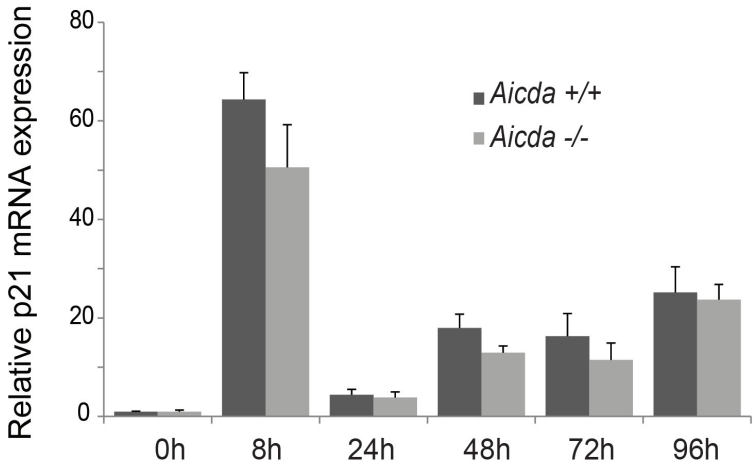


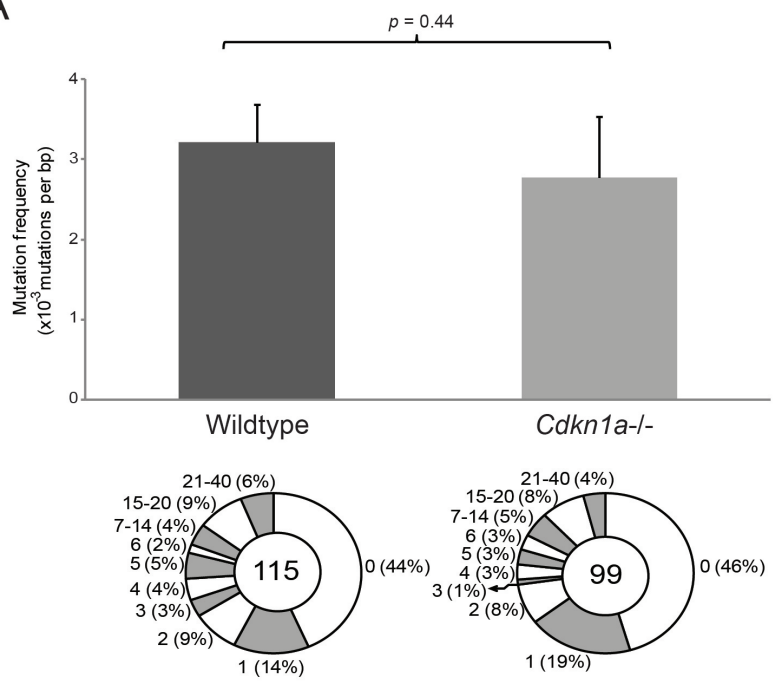
Figure 1. p21 expression is not regulated by AID. B cells were isolated from splenocytes and stimulated with LPS and IL-4 for the time periods indicated. Relative p21 mRNA expression was determined after normalizing to β-actin. Fold-differences are shown by setting the p21 expression level at time 0 hr to one. There are no significant differences in p21 expression between AID-sufficient (*Aicda*$^{+/+}$) and AID-deficient (*Aicda*$^{-/-}$) B cells at all time periods as indicated by two-tailed unpaired Student's *t*-tests, $p \geq 0.1$. Comparison of p21 expression between each time point was only statistically different between time points 0, 8, and 24 hours ($p = 3.6 \times 10^{-5}$ comparing time 0hr with 8hr, $p = 0.006$ comparing time 0hr with 24hr, $p = 0.0015$ comparing time 24hr with 48hr). The *p* values between 48hr and 72hr was 0.6, and the *p* value between time 48hr and 96hr was 0.09. Three independent experiments were performed from 3 *Aicda*$^{-/-}$ and 2 *Aicda*$^{+/+}$ mice. Bar graphs represent the mean values from three independent experiments. Error bars represent standard deviations (SD).

2.2 p21 is dispensable for SHM.

To investigate whether p21 deficiency had a significant effect on SHM frequency, I isolated activated B cells from Peyer's patches of *Cdkn1a*$^{-/-}$ mice and *Cdkn1a*$^{+/+}$ or *Cdkn1a*$^{+/-}$ littermate controls. Because the results from *Cdkn1a*$^{+/+}$ and *Cdkn1a*$^{+/-}$ mice were similar (*Supplementary Table 1*) I pooled them and refer to these as wildtype in these SHM experiments. Activated B cells from Peyer's patches are activated naturally by gut-associated antigens (385) and activated B cells were isolated as B220^{+}PNA^{high}. To assess Ig locus SHM, DNAs containing the Jh2-Jh4 intronic region of the Ig locus were amplified to monitor mutations that are not located in coding regions and, therefore, are not affected by antigenic selection. A total of 115 clones from wildtype mice and a total of 99 clones from *Cdkn1a*$^{-/-}$ mice were analyzed for SHM by sequence analyses. I found no differences in SHM frequency or in mutation patterns when comparing *Cdkn1a*$^{-/-}$ and wildtype mice (Figure 2). The mutation frequency in *Cdkn1a*$^{-/-}$ mice (2.77×10^{-3}) was somewhat lower than in wildtype mice (3.21×10^{-3}), but this difference was not statistically significant (Figure 2A, top). In addition, the percentage of mutated clones was similar between wildtype (56%) and *Cdkn1a*$^{-/-}$ (54%) mice, indicating that p21 deficiency did not lead an increase in the number of B cells undergoing SHM (Figure 2A, bottom). Furthermore, the numbers of mutations per clone in *Cdkn1a*$^{-/-}$ and wildtype B cells were similar; about 19% of the clones in the wildtype mice, and 17% of the clones in the *Cdkn1a*$^{-/-}$ mice exhibited 7-40 mutations (Figure 2A, bottom).

My results also indicated that the SHM mutation pattern was the same in both wildtype and *Cdkn1a*$^{-/-}$ mice (Figure 2B). The frequency of mutations at each base (A, T, G, C) was not significantly altered in *Cdkn1a*$^{-/-}$ mice (Figure 2C). About half of the mutations were transition mutations (wildtype 51.3%, *Cdkn1a*$^{-/-}$ 49.9%) and the other half were transversion mutations (wildtype 48.7%, *Cdkn1a*$^{-/-}$ 50.1%) (Table 1). Most importantly, mutations at A:T bp, which can be generated by translesional DNA pol η, were not affected by the absence of p21 (wildtype 62.8%, *Cdkn1a*$^{-/-}$ 61.9%), and the percent of mutations at DNA pol η hotspots were not statistically different between the two mouse strains (Table 1).

A



B

Mutation number *(N mutations)*

	To	A	T	G	C	Sum
Wildtype	A		63	115	45	**223**
	T	41		22	43	**106**
	G	65	20		24	**109**
	C	23	46	17		**86**
	Total					**524**
Cdkn1a-/-	A		55	75	30	**160**
	T	35		13	29	**77**
	G	47	22		18	**87**
	C	7	40	12		**59**
	Total					**383**

Relative frequency *(% from total)*

	To	A	T	G	C	Sum
Wildtype	A		12.0	21.9	8.6	**42.6**
	T	7.8		4.2	8.2	**20.2**
	G	12.4	3.8		4.6	**20.8**
	C	4.4	8.8	3.2		**16.4**
	Total					**100**
Cdkn1a-/-	A		14.4	19.6	7.8	**41.8**
	T	9.1		3.4	7.6	**20.1**
	G	12.3	5.7		4.7	**22.7**
	C	1.8	10.4	3.1		**15.4**
	Total					**100**

C

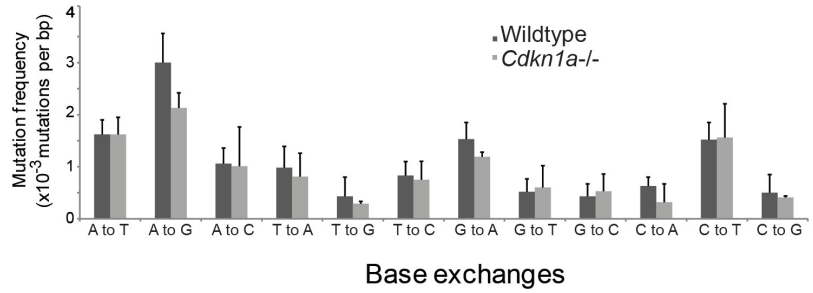


Figure 2

Figure 2. p21 is dispensable for SHM. (A) Top: SHM frequency was determined from activated B cells that were isolated from Peyer's patches and the Jh2-Jh4 intronic region was amplified. Three independent experiments (4 mice per genotype per experiment) were performed. Mutation frequency was determined by dividing the total number of mutations to the number of total bp analyzed. Bar graphs represent the mean values from three independent experiments. Error bars represent standard deviations (SD). *p* values calculated using two-tailed unpaired Student's *t*-tests. Bottom: Pie charts representing data for SHM. The center of the pie indicates the number of clones sequenced. The numbers around the pie chart represent the number of mutations. The numbers inside the parenthesis are the percentages of clones containing the indicated number of mutations. **(B)** SHM spectrum. Total number of mutations from three independent experiments combined are represented in the tables on the right whereas the tables on the left indicate the percentages of mutations at each bp. **(C)** To simplify the comparison of mutation patterns, the SHM frequency at each bp, corrected for base composition, is shown. The results were confirmed by the SHMTool webserver program (http://scb.aecom.yu.edu/cgi-bin/p1). There were no statistical differences found by two-tailed unpaired Student's *t*-tests. Error bars represent SD.

Table 1. SHM summary (Jh2-Jh4 Peyer's Patches)

	Wildtype	*Cdkn1a*-/-	*p* value
Total number of base pairs analyzed[a]	171,580	147,708	
Total number of mutations[b]	524	383	
Overall mutation frequency[c]	3.21×10^{-3}	2.77×10^{-3}	0.44
Mutated sequences (%)	56.0	54.0	
Total transition mutations (%)	51.3	49.9	0.27
Total transversion mutations (%)	48.7	50.1	0.54
A/T mutations (%)	62.8	61.9	0.58
% Transitions	30.2	27.2	0.24
% Transversions	32.6	34.7	0.59
G/C mutations (%)	37.2	38.1	0.58
% Transitions	21.2	22.7	0.70
% Transversions	16.0	15.4	0.84
AID hotspots (%)[d]			
WRC/	8.8	10.2	0.55
/GYW	8.4	11.0	0.06
Pol η hotspots (%)[d]			
WA/	31.5	26.4	0.29
/TW	12.4	12.8	0.99

[a] 1.4 kb Jh2-Jh4 intronic region was amplified from activated B cells and sequenced bidirectionally. Three independent experiments (4 mice per genotype per experiment) were performed.

[b] Clones containing the same mutations were counted once.

[c] Mutation frequency was calculated by dividing the total number of mutations by the total number of base pairs analyzed. The results were confirmed by using the SHMTool webserver (http://scb.aecom.yu.edu/cgi-bin/p1).

[d] Underlined site of the motif is scored. W = A/T, R = A/G, Y = C/T. Motifs were identified using SHMTool webserver.

In order to investigate whether p21 had a role in CSR, I tested for the ability of *Cdkn1a*$^{-/-}$ B cells to class switch to IgG1, IgG3, IgG2a, and IgG2b when stimulated *ex vivo*. The percentage of CSR in both wildype and *Cdkn1a*$^{-/-}$ B cells were similar (Figure 3), indicating that, as with SHM, p21 does not function in CSR. Based on these data, I conclude that p21 does not play a role in regulating Ab gene diversification in B cells.

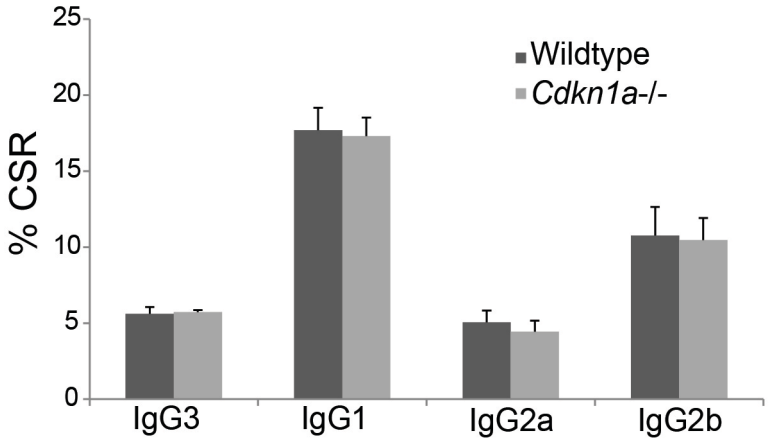


Figure 3. p21 deficiency does not affect CSR to IgG3, IgG1, IgG2a, and IgG2b. B-cells from *Cdkn1a*$^{-/-}$ and *Cdkn1a*$^{+/+}$ mice were stimulated with the appropriate cytokines (see materials and methods) for 4 days to induce CSR. The percentage of cells that switched to the indicated isotypes were determined by flow cytometry. Three independent experiments (1 mouse per genotype per experiment) were performed. Bar graphs represent the mean values from three independent experiments. Error bars represent SD.

3. Supplementary Data

Supplementary Table S1. SHM summary between *Cdkn1a*+/+ and *Cdkn1a*+/- mice (Jh2-Jh4 Peyer's Patches).

	experiment 1 *Cdkn1a*+/+	experiment 2 *Cdkn1a*+/+	experiment 3 *Cdkn1a*+/-
Total number of base pairs analyzed [a]	52,220	95,488	23,872
Total number of mutations [b]	188	256	80
Overall mutation frequency [c]	3.6×10^{-3}	2.7×10^{-3}	3.3×10^{-3}
Mutated sequences (%)	57	53	69
Total transition mutations (%)	50	51	55
Total transversion mutations (%)	50	49	45
A/T mutations (%)	63	61	65
G/C mutations (%)	37	39	35

[a] 1.4 kb Jh2-Jh4 intronic region was amplified from activated B cells and sequenced bidirectionally. Three independent experiments (4 mice per genotype per experiment) were performed.
[b] Clones containing the same mutations were counted once.
[c] Mutation frequency was calculated by dividing the total number of mutations by the total number of base pairs analyzed. The results were confirmed by using the SHMTool webserver (http://scb.aecom.yu.edu/cgi-bin/p1).

4. Discussion

My results show that, in activated B cells, p21 deficiency does not alter the amount of AID-induced mutagenesis that occurs within the Igh locus during SHM and CSR. This contrasts with the known role of p21 (together with the tumor-suppressor protein, p53) in controlling DNA-damage-mediated mutagenesis in other types of mammalian cells (130). Based on previous studies, it could be suggested that the lack of a p21 effect on SHM could result from a specific down-regulation of p21 in these cells (155, 156). These studies, however, did not address the *in vivo* role of p21 in B cells, particularly whether down-regulation of p21 might be a mechanism to promote SHM. I find that, although p21 transcript levels do decrease in the period between 8 to 24 hours after B cell stimulation, these levels recover during the period 48 to 96 hours after stimulation, at the times when AID-induced DNA damage repair leads to CSR events (386). Furthermore, I have shown that the p21 expression pattern does not differ between wildtype and AID-deficient mice, indicating that regulation of p21 is independent of AID activity. I suspect that reduction of functional p21 expression may be relevant for cell cycle control as suggested previously (155, 156), but not for SHM.

I also find that, at least for the Igh loci, which are poised to accrue mutations, p21 deficiency does not result in an increase in SHM as would be predicted if p21 had a negative regulatory role. My results are consistent with the

findings from a recent study that investigated the role of p53 and p21 in SHM (159). This study shows that the mutation spectrum is altered in p53 deficient B cells. This change, however, was not detected in B cells lacking p21 or in p53/Ung double deficient B cells (159). Based on these findings, it was concluded that p21 does not function in SHM. Perhaps it is more likely that p21 activity is excluded from the Igh locus during SHM and, therefore, p21 deletion has no effect on the frequency of Igh locus SHM. However, I do not know whether p21 expression might, at later time points, function to prevent mutagenesis at non-Ig genes that have also been shown to accumulate AID-induced DNA damage but that are repaired in an error-free manner (109). Indeed, it has been shown that p21 can interfere with PCNA ubiqutination and the ability of PCNA to load translesional DNA polymerases, such as Pol η, in human cell lines (131, 133). Furthermore, PCNA mutants that are unable to become monoubiquitinated show discernable effects on SHM, particularly at bases that are targeted by Pol η (120, 121). Therefore, it is possible that p21 interaction with PCNA may promote error-free repair of non-Ig genes, but it is clear from our results that p21 does not affect the SHM process at the Igh loci.

Since p21 can function in promoting error-free DNA repair by regulating DNA polymerase loading onto PCNA (131, 133), I also considered that the absence of p21 might result in the inhibition of faithful DNA repair of Ig switch regions and could create more double stranded DNA (dsDNA) breaks as reported for B cells lacking the error-free DNA polymerase Polβ (387).

Therefore, an increase in CSR might be anticipated in p21-deficient B cells. I tested this hypothesis and found that CSR is not affected in p21-deficient B cells, indicating that p21 does not play a role in AID-mediated Ig gene diversification.

CHAPTER 6

DISCUSSION

1. Translocation of an antibody transgene requires AID and occurs by interchromosomal switching to all switch regions except the mu switch region.

Aberrant CSR has been linked to translocations that occur between the Igh locus and proto-oncogenes, such as c-myc (341, 342, 350, 351). It has been well established now that the initiating factor in these translocations is AID-induced DNA damage at both the Igh locus and the c-myc locus (161, 356-359, 370). Here, I have shown that translocations between an Ig transgene (VV29) and the Igh locus are also AID-dependent, although a very low frequency of AID-independent translocations are also detected in some, but not all, mice that were immunized with a highly immunogenic substance, complete Freund's adjuvant (CFA). More interestingly, the translocation events can occur *in vitro* by stimulating primary B-cells in culture and the *in vitro* translocation frequency is estimated to be 3%. This indicates that the VV29 mouse could potentially be used as model to investigate the mechanism(s) of interchromosomal recombinations since, unlike other mouse models that have been used (349, 356, 357), the VV29 mouse is not missing any essential genes that could cause genomic instability, and further, *in vitro* experiments would be faster to test many genetic alterations.

Recently, it was reported that translocations, particularly Igh/c-myc translocations, during CSR are more likely to occur when the NHEJ pathway is defective, i.e. the A-EJ pathway is more prone to causing translocations (355). It would be interesting to investigate whether the frequency of translocation between the VV29 transgene and the Igh locus also would increase in the absence of NHEJ factors. This would answer the question on whether Igh/transgene tranlocations, similarly to Igh/proto-oncogene translocations, occur as a result of "*aberrant CSR*" or whether they are similar to intrachromosomal (cis) CSR, and thus, rely on the general CSR machinery. Because the VV29 transgene resembles the Igh locus, and the frequency of Igh/transgene translocations are orders of magnitude higher than Igh/c-myc translocations (161), I suggest that the molecular machinery for VV29 translocations may be different from the molecular machinery that causes Igh/c-myc translocations. This would explain the apparent discrepancy related to the role of the Igh 3'RR enhancers in translocations. Although, it was suggested that they are important in protecting against translocations between the Igh locus and an Ig transgene (181), another study showed that Igh/c-myc translocations occur regardless of the presence or absence of the 3'RR enhancers (349).

VV29 translocation into the Igh locus further resembles interchromosomal CSR in that the transgene can be joined with at least Cγ3, and Cγ1 endogenous C-regions. Surprisingly, trans-switching between the transgenic Sμ and the endogenous Sμ regions was not detected by the RT-PCR/Southern blot assays.

Based on later cloning/sequencing analyzes from activated ($B220^{+}$ PNA^{high}) VV29 B-cells, 0.69% of the VV29 VDJ-Cμ clones (2 clones out of 289 total clones analyzed), were associated with the endogenous Cμ gene, i.e. trans-Sμ-to-Sμ recombination occurred. This is almost 5 fold lower than 3.3% translocation frequency that occurs between the transgene and endogenous Cγ genes (377), which implies that, during interchromosomal CSR, trans-Sμ-to-Sμ recombination may occur, albeit, at lower frequency than trans-Sμ-to-Sx (x being any S region other than Sμ). This makes biological sense, in that, trans-CSR between two homologous Cμ regions does not result in a change in isotype, therefore, may have been selected against during the course of the immune system development. Nevertheless, since these results rely on a single nucleotide difference between the Cμ regions, this area of research needs to be further investigated with a better mouse model. The question of whether, at the endogenous Igh locus, S regions on homologous chromosomes, can recombine during trans-CSR is important to address and may shed more light into the regulation of CSR, especially since trans-CSR has been shown to significantly contribute to the generation of Ab classes.

2. AID-induced mutagenesis occurs in an immunoglobulin transgene that is missing Igh regulatory *cis* elements downstream of the Cmu gene.

The VV29 transgene was further used to investigate the need for AID in transgene V-gene conversion, and to investigate whether the transgene was capable of accumulating AID-induced mutations, even though, the transgene does not have Igh 3'RR enhancers that were previously shown to be important for transgene SHM (178, 181). Based on RT-PCR/Southern blot assays, Ig V-gene conversion was not detected at transgene locus, although, the transgene VDJ region did accrue AID-induced DNA damage. Furthermore, none of the 251 clones that were analyzed in VV29 mice exhibited gene conversion. These results indicate the DNA damage at the transgene locus may not be extensive enough to activate the gene conversion pathway.

Using VV29:Ung$^{-/-}$:Msh2$^{-/-}$ mice, it was shown that the transgene locus, as compared to the endogenous Igh locus, does indeed have lower AID activity. Surprisingly, the mutation frequency at both loci decreases in the absence of Ung and Msh2 proteins, indicating that these proteins have mutagenic roles at Ig or Ig-like loci. Because the VV29 transgene has the Eμ intronic enhancer and a 600 bp Sμ region, it is possible that these *cis* elements make the transgene more prone to accumulating a higher degree of AID-induced damage than compared to non-Ig genes that have also been reported to generate AID-induced DNA damage but are repaired in an error-free manner (109). These results lead to the

hypothesis that perhaps the degree of AID-induced damage may be one (of many) mechanism(s) of determining whether the repair is by error-prone versus error-free. This hypothesis can be tested by generating mice that have some portion of S regions near non-Ig gene(s) that have been previously shown to be targeted by AID but repaired by error-free mechanisms and examining whether the addition of the S region results in error-prone DNA repair.

3. p21 is dispensable for AID-mediated class switch recombination and mutagenesis of immunoglobulin genes during somatic hypermutation.

In an attempt to investigate the regulatory factors that may be involved in promoting error-prone versus error-free repair during SHM, p21 was investigated because of its role in affecting monoubiquitination of PCNA, and therefore, limiting mutagenesis (130, 131, 133). The role of p21 was found to be dispensable during SHM of Ig genes. Also, similar levels of p21 mRNA in stimulated B-cell cultures in both wildtype and $AID^{-/-}$ mice was observed indicating that p21 levels are not regulated by DNA damage. p21 expression peaked at 8 hours post-stimulation, however, by the time AID is expected to be expressed (24-48hours post-stimulation) (195, 386), the levels of p21 mRNA were drastically reduced. Since high levels of DNA damage occur at Ig loci during B-cell activation, it makes sense to limit p21 levels, and indeed this was proposed before (155, 156). These results indicate that p21 is probably

regulated during SHM not because it may function to suppress mutagenesis, but rather to promote proper B-cell cycling. Although, it remains to be determined whether p21 promotes error-free repair at non-Ig loci.

Recently, some studies have shown that p53 can suppress DNA damage at S regions during CSR and regulate mutagenesis during SHM (160). Furthermore, it has been known for a while now that p53 protects against Igh/c-myc translocations (161). Because many DNA repair factors, such as PCNA, error-free DNA Pol β, BER and MMR proteins, to name a few, can be regulated by p53 (149, 150, 152-154, 388-390), the next set of experiments should focus towards understanding how each of these factors function at different chromosomal locations and cell cycle stages.

4. Conclusion

Using an Ig transgenic mouse model, I have shown that trans-CSR between the endogenous Igh locus and the transgene locus occurs relatively frequently *in vitro*, although, trans-switching between endogenous Sμ and the transgene Sμ, i.e. between homologous S regions, are not readily detected. Furthermore, Ung and Msh2 proteins, similar to the endogenous Igh locus, had a mutagenic function at the transgene locus. These results suggest that Ig loci may contain the necessary *cis* elements that make it prone to error-prone repair and raises

many interesting questions about the regulation of CSR and SHM. For example, since trans-CSR has been documented, is trans-switching between homologous S regions at the endogenous Igh loci avoided and how does this fit into the chromosomal looping structure that was reported to protect against interchromosomal recombinations (250)? It is possible that trans-CSR is part of the "aberrant CSR" and thus is similar the Igh/c-myc translocations, i.e. occurs via the A-EJ pathway, therefore, may not contribute significantly to CSR when the NHEJ pathway is intact. Furthermore, what elements at the Igh locus make the locus prone to accrue mutations? Perhaps the presence of the Eμ intronic enhancer in the VV29 transgene, makes the transgene a better target of more AID deamination, as compared to non-Ig genes, and therefore, the extent of DNA damage may activate/modulate specific DNA repair factors to function differently at specific chromosomal loci. Finally, the Ig V-gene conversion results raises the question of how much the gene conversion pathway contributes to Ig diversification. These are questions that may be investigated in the future.

I also showed that p21 was dispensable for SHM and CSR, although, p21 in other types of mammalian cells, using plasmid-based assays to measure mutagenesis, had a role in protecting against mutagenesis (130, 131). This again raises the question of how are proteins regulated such that their function varies at different chromosomal loci.

Together, the results presented in this thesis suggest that *cis* elements present on the Igh locus may function to maximize the B-cell responses to antigens.

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